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PhD Thesis

Dr Gareth Jones

**Optimising islet transplantation:
experimental and clinical observations.**

Royal Free and University College Medical School,
London, NW3 2PF.

Supervisors

Prof S H Powis

Dr M Press

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Abstract

Clinical islet transplantation is an accepted treatment for the severe secondary complications of type 1 diabetes but is limited by the number of donors needed to achieve insulin independence. The requirement for multiple donors is probably due to the loss of up to 60% of islet mass after transplantation from hypoxia, caused by a lack of functional vasculature. Revascularisation of the transplanted islet does occur but takes up to 14 days.

The aim of this project was to investigate the revascularisation process and devise a method of manipulating islets in order to improve islet engraftment. The scientific research was supplemented by the establishment of clinical transplantation.

A syngeneic intraportal model of islet transplantation was used to explore the revascularisation of islets and develop a novel dual endothelial/insulin staining technique. The experiments show clear observation of revascularisation with quantifiable changes in vascular density and islet composition. Interestingly, the vascular density of transplanted islets was greater than native islets.

Pre treatment of isolated islets with desferrioxamine explored as a method for up regulating angiogenic growth factors. This method produced a sustained increase in VEGF expression that did not adversely affect islet viability or secretory function but did not improve clinical outcome post transplant.

Three diabetic patients were transplanted with human islets and the outcome, clinical course and issues related to organ donation and processing are and also discussed.

Islet revascularisation is quantifiable and can be explored to examine the effect of manipulating engraftment. Iron depletion offers a simple method for manipulating islets prior to transplantation but further work is necessary to establish if there is any improvement in islet engraftment with this technique. Clinical islet transplantation programmes within the UK are achievable but further progress, collaboration and funding are required to establish islet transplantation as a routine treatment.

Table of contents

Index of tables and figures	10
Abbreviations	14
Chapter 1 - Introduction	16
1.1 Impact of diabetes	16
1.2 Treatment options for diabetes	18
1.3 Clinical islet transplantation	19
1.4 Clinical islet transplantation in the United Kingdom	21
1.4.1 History and current situation of UK clinical islet transplantation.....	21
1.4.2 Funding and staffing of UK islet transplant centres.....	21
1.4.3 Isolation and purification of human islets for transplantation	22
1.4.4 Donor pancreases for islet isolation	23
1.4.5 Patient selection and management	24
1.5 Insulin independence and peri-transplant islet loss.....	26
1.6 Native islet structure and vasculature.....	27
1.7 Transplanted islet vasculature.....	28
1.7.1 Structure of transplanted islet vasculature	28
1.7.2 Origin of transplanted islet endothelium.....	28
1.8 Control of the revascularisation process.....	30
1.9 Manipulation of the revascularisation process.....	32
1.10 Hypoxia sensing and hypoxia inducible factor.....	33
1.11 Hypoxia sensing and desferrioxamine.....	36
1.12 Experimental models of islet transplantation.....	38
1.13 Purpose of research.....	39
1.14 Hypothesis.....	41
1.15 Aims of the research described in this thesis	41
Chapter 2 - Materials and methods.....	42
2.1 Commonly used solutions	42
2.2 Common in-vivo techniques.....	42
2.2.1 Experimental subjects	42
2.2.2 Isolation of rat islets.....	42
2.2.3 Measurement of blood glucose	43
2.2.4 Induction of diabetes.....	44
2.2.5 Transplantation of islets	44
2.2.6 Intra-peritoneal glucose tolerance test	46
2.2.7 Retrieval of liver and pancreas.....	46
2.2.8 Fixation and embedding of livers.....	47
2.2.8a Paraffin-embedded sections	47
2.2.8b Frozen livers for in situ	47
2.3 Histological analysis of transplanted islets	48
2.3.1 Haematoxylin and eosin staining	48
2.3.2 Immunohistochemistry techniques	48
2.3.3 Combined endothelial and insulin staining.....	49

2.3.4 Quantitative histological analysis	51
2.3.4a Scaling of images	51
2.3.4b Measurement of engrafted islet area	51
2.3.4c Measurement of percentage vascular area of islets	51
2.3.4d Measurement of vascular density	52
2.3.4e Calculation of the branching index	52
2.3.4f Measurement of vessel size	53
2.3.5 Fluorescent in-situ hybridisation of the Y chromosome	54
2.4 In-vitro desferrioxamine treatment studies	55
2.4.1 Effect of desferrioxamine treatment on expression of VEGF and bFGF protein in cultured islets	55
2.4.2 Measurement of angiogenic growth factors by ELISA	56
2.4.2a Measurement of VEGF	56
2.4.2b Measurement of bFGF	56
2.4.2c Measurement of HIF-1 α	56
2.4.2d Measurement of total protein concentration from cell lysates	57
2.4.3 Effect of desferrioxamine treatment on angiogenic factor gene expression ...	57
2.4.4 Effect of desferrioxamine treatment on islet viability	57
2.4.4a Flow cytometry method on dispersed islets	58
2.4.4b Direct visualisation of whole islets	58
2.4.5 Effect of desferrioxamine treatment on apoptosis of islets	59
2.4.5a Isolation and staining of peripheral blood mononuclear cells	59
2.4.6 Effect of desferrioxamine treatment on the glucose related function of islets	61
2.4.6a Glucose stimulation test	61
2.4.6b Assessment of insulin content of islets	61
2.5 RNA molecular techniques	62
2.5.1 Isolation of RNA	62
2.5.2 Analysis of RNA concentration and purity	62
2.5.3 DNase treatment of RNA samples	63
2.5.4 Formation of cDNA	63
2.5.5 Design of real time PCR primers	63
2.5.7 Real time PCR estimation of gene quantification	65
2.5.7 Validation of PCR primers	67
2.5.7a Isolation of rat genomic DNA	67
2.5.7b Validation of primers	68
2.5.7c Assessment of PCR products	68
2.5.8 Analysis of amplification plots and relative fold gene expression	69
2.5.8a Calculation of threshold cycle (Ct) values	69
2.5.8b Calculation of intra sample differences in gene expression (Δ Ct)	70
2.5.8c Calculation in inter sample differences in gene expression ($\Delta\Delta$ Ct)	71
2.5.8d Calculating the relative fold change in gene expression	72
2.6 DNA molecular techniques	73
2.6.1 IMAGE clone selection	73
2.6.2 IMAGE clones chosen	73
2.6.3 Rat Y chromosome probe	73
2.6.4 Broth amplification of plasmids	74

2.6.5 Isolation of plasmid DNA	74
2.6.6 Glycerol stock of plasmids.....	74
2.6.7 Digestion of plasmids.....	75
2.6.8 Check of plasmids by PCR prior to broth amplification.....	75
2.6.9 Polymerase chain reaction (PCR) amplification	75
2.6.10 Purification of PCR product.....	75
2.6.11 Estimation of PCR product length	76
2.6.12 Estimation of DNA concentration and purity	76
2.6.13 Sequencing of PCR products	76
2.6.14 Confirmation of sequences of PCR products	77
2.7 In vivo desferrioxamine treatment studies	78
2.7.1 Effect of transplanting desferrioxamine treated islets on reversal of diabetes.....	78
2.7.2 Histological analysis of desferrioxamine treated and control islets.....	78
2.8 Clinical islet transplantation	79
2.9 Statistical analysis	79
 Chapter 3 – Results: Validation and new techniques	 80
3.1 Introduction	80
3.2 Validation of common in-vivo techniques.....	83
3.2.1 Validation of capillary blood glucose measurements	83
3.2.2 Validation of diabetes induction.	84
3.2.3 Validation of rat islet isolation.....	85
3.2.4 Validation of non selective intra-portal transplantation.....	86
3.2.5 Validation of sex mismatched right branch intra-portal transplantation.....	87
3.2.6 Conclusions of in vivo validation	89
3.3 Development of histological techniques	90
3.3.1 Tissue sectioning and mounting.....	90
3.3.2 Histology assessment of intra-portal transplanted islets	91
3.3.3 Conclusions of histological optimisation	96
3.4 Development of in-vitro assays for growth factor protein expression	97
3.4.1 Assay of VEGF protein expression.....	97
3.4.2 Assay of bFGF protein expression.....	97
3.4.3 Assay of HGF protein expression	99
3.4.4 Assay of HIF-1 α concentrations	99
3.4.5 Conclusions from in-vitro assay of growth factor protein expression	100
3.5 Development of molecular techniques.....	101
3.5.1 Isolation of RNA	101
3.5.2 Additional primer sets	102
3.5.3 Formation of cDNA	103
3.5.4 DNA contamination	104
3.5.4a Reduction of exogenous DNA contamination.....	105
3.5.4b Reduction of endogenous DNA contamination	105
3.5.5 Evaluation of primers.....	108
3.5.6 Selection of a control primer pair.....	113
3.5.7 Conclusion from the development of molecular techniques.....	114
3.6 Development of islet apoptosis assay	115

3.7 Assessment of islet viability	120
3.7.1 Flow cytometry method	120
3.7.2 Intact islets	121
3.7.3 Conclusions from assessment of islet viability	121
3.8 Development of in-situ techniques	123
3.8.1 RNA in situ hybridisation	123
3.8.2 Fluorescent in-situ hybridisation of the Y chromosome	124
3.8.3 Conclusions from in-situ hybridisation techniques	128
3.9 Discussion	130
3.9.1 Islet transplant model	130
3.9.2 Growth factor analysis	131
3.9.3 Islet integrity analysis	132
3.9.4 In-situ techniques	134
3.9.5 Conclusions	134
Chapter 4 – Results: The revascularisation of intra-portally transplanted islets	135
4.1 Introduction	135
4.2 Change in clinical parameters post sex-mismatched right branch transplantation	137
4.3 Qualitative histological analysis of native islets	139
4.4 Qualitative histological analysis of engrafting transplanted islets	145
4.4.1 Recovery of islets from transplanted recipients	145
4.4.2 The day of transplantation	146
4.4.3 One day post transplantation	148
4.4.4 Three days post transplantation	150
4.4.5 Five days post transplantation	153
4.4.6 Seven days post transplantation	155
4.4.7 Fourteen days post transplantation	157
4.4.8 Thirty days post transplantation	159
4.5 Quantitative analysis of native and transplanted islets	161
4.5.1 Islet area in native and transplanted islets	161
4.5.2 Percentage of islet comprised of endothelium	161
4.5.3 Vascular density measurements	162
4.5.3a Branch counting method	162
4.5.3b Grid method	162
4.5.4 Branching index	165
4.6 Discussion	166
4.6.1 Assessment of native islets	166
4.6.2 The change in islet area after transplantation	167
4.6.3 The revascularisation of transplanted islets	168
4.7 Conclusions	170
Chapter 5 – Results: In-vitro desferrioxamine treatment studies	171
5.1 Introduction	171
5.2 Effect of desferrioxamine treatment on protein expression of growth factors	173

5.2.1 VEGF protein expression from desferrioxamine treated islets after overnight culture.....	174
5.2.2 bFGF protein expression from desferrioxamine treated islets after overnight culture.....	175
5.2.3 VEGF protein expression from desferrioxamine treated islets after culture in M199	176
5.3 Effect of desferrioxamine treatment on gene expression of growth factors ..	177
5.3.1 Change in expression of VEGF mRNA with desferrioxamine treatment.....	178
5.3.2 Change in expression of HGF mRNA with desferrioxamine treatment	179
5.3.3 Change in expression of bFGF mRNA with desferrioxamine treatment.....	179
5.3.4 Change in expression of Ang1 mRNA with desferrioxamine treatment.	179
5.3.5 Change in expression of endostatin mRNA with desferrioxamine treatment	179
5.4 Effect of desferrioxamine treatment on islet viability	180
5.5 Effect of desferrioxamine treatment on islet apoptosis	182
5.6 Effect of desferrioxamine treatment on glucose stimulated insulin release..	184
5.6.1 Glucose stimulation tests	184
5.6.2 Insulin content of islets	185
5.7 Discussion	187
5.7.1 Effect of desferrioxamine treatment on angiogenic growth factor expression	187
5.7.1a Expression of vascular endothelial growth factor	187
5.7.1b Expression of Hepatocyte growth factor.....	191
5.7.1c Expression of basic fibroblast growth factor.....	192
5.7.1d Expression of angiopoietin 1.....	194
5.7.1e Expression of endostatin	196
5.7.1f Angiogenic factor expression and effect	197
5.7.2 Effect of desferrioxamine treatment on islet viability.....	198
5.7.3 Effect of desferrioxamine treatment on islet apoptosis.....	199
5.7.4 Effect of desferrioxamine treatment on insulin secretion	201
5.7.4 Effect of desferrioxamine treatment on islet insulin content	202
5.8 Conclusions	203
Chapter 6 – Results: In-vivo desferrioxamine treatment studies	204
6.1 Introduction	204
6.2 Effect of desferrioxamine treatment on ultra-marginal (350 islet) syngeneic islet transplants	206
6.2.1 Change in blood glucose after 350 islet transplant	206
6.2.2 Change in body weight after 350 islet transplant.....	209
6.3 Effect of desferrioxamine treatment on marginal (500 islet) syngeneic islet transplants	210
6.3.1 Change in blood glucose after 550 islet transplants.....	210
6.3.2 Change in body weight after 550 islet transplant.....	212
6.4 Histological analysis of desferrioxamine treated and control islets post ultra-marginal (350 islet) transplants.	214
6.4.1 Gross histological appearance of transplanted control and desferrioxamine treated islets.....	214

6.4.2 Quantitative histological analysis of desferrioxamine treated and control islets	219
6.4.2a Analysis of islet area	219
6.4.2b Analysis of percentage vascular area and vascular density	220
6.4.2c Analysis of branching index.....	221
6.4.2d Analysis of transplanted islet vessel size	222
6.5 Discussion	223
6.5.1 Suitability of transplant model.....	223
6.5.1a Induction of diabetes	223
6.5.1b Measurement of blood glucose	225
6.5.1c Islet dose.....	226
6.5.2 Histological outcomes.....	227
6.6 Conclusions	231
Chapter 7- Results: Clinical islet transplantation	232
7.1 Introduction	233
7.2 History of the Royal Free clinical islet transplant programme	234
7.3 Donor pancreases for islet isolation	234
7.4 Pancreatic islet isolation	236
7.4.1 Islet yield from processed pancreases	236
7.4.2 Cooling of the Cobe 2991 centrifuge.....	237
7.5 Clinical islet transplantation	243
7.5.1 Islet recipient number 1.....	243
7.5.2 Islet recipient number 2.....	247
7.5.3 Islet recipient number 3.....	250
7.6 Discussion	253
7.6.1 Introduction.....	253
7.6.2 Clinical islet transplantation.....	253
7.6.3 Complications of islet transplantation.....	255
7.7 Conclusions	257
Chapter 8 – Discussion	258
8.2 The revascularisation of intra-portal transplanted islets	260
8.2.1 Introduction.....	260
8.2.2 Models of transplanted islet revascularisation	260
8.2.3 Revascularisation of intra-portal transplanted islets	261
8.2.4 Methods for assessing vascular density	264
8.2.5 Vascular density in native and transplanted islets.....	266
8.2.6 Conclusions	270
8.3 Desferrioxamine treatment of islets	271
8.3.1 Effect of desferrioxamine treatment on isolated islets.....	271
8.3.2 Effect of desferrioxamine on transplanted islets.....	273
8.3.2a Action of desferrioxamine and availability of iron	273
8.3.2b Administration of desferrioxamine	274
8.3.2c The importance of VEGF to islet engraftment.....	274
8.3.3 Conclusions	275

8.4 Clinical islet transplantation	276
8.4.1 Introduction	276
8.4.2 Islet transplantation in the UK	276
8.4.3 The place of islet transplantation in management of diabetic patients	277
8.4.5 The requirement for insulin independence.....	279
8.4.6 Conclusions	283
8.5 Overall conclusions	284
Appendix 1: Commonly used solutions	285
Appendix 2: NICE guidance on pancreatic islet transplantation.....	289
Appendix 3: Local ethic committee submission	291
Bibliography	302

Index of tables and figures

Chapter 1	16
Figure 1.1: Cost of diabetes in the United States	17
Figure 1.2: Structure and vascular supply of native islets	27
Figure 1.3: The metabolism of Hypoxia Inducible Factor alpha	34
Figure 1.4: Caudal view of rat liver	38
Chapter 2	42
Figure 2.1: Method for right branch transplantation of islets.	46
Table 2.1: Antigen retrieval and dilution of antibodies for immunohistochemistry.....	49
Figure 2.2: Formation of the tricolour image for the dual BS-1 and insulin technique...50	
Figure 2.3: Methodology for assessing islet area and vascular density of islets	53
Table 2.2: Primer sequences for gene analysis by real time PCR.....	66
Table 2.3: Composition of cDNA amplifications and control amplifications	66
Figure 2.4: Amplification data expressed as signal against cycle number when displayed using ABI Prism 7000 software.	70
Chapter 3	80
Figure 3.1: Correlation between blood glucose concentrations measured by the Accu-Check Advantage II glucometer and plasma glucose concentrations measured by the Cobas Mira auto-analyser.	84
Table 3.1: Comparison of islet yield when using different volumes of buffer infused with collagenase.	86
Table 3.2: Weight and blood sugar of rats prior to and after transplantation during experiments to validate sex matched and sex mismatched transplant model	87
Figure 3.2: Change in blood glucose after transplant in 5 diabetic female recipients receiving male islets transplanted to the main branch portal vein.	88
Figure 3.3: Serial sections of recipient rat liver stained by immunohistochemistry	92
Figure 3.4: Neuraminidase antigen retrieval optimisation.....	94
Table 3.3: Experimental design for optimising the concentration of biotinylated BS-1 and streptavidin-FITC.	95
Figure 3.5: Mean bFGF concentration in supernatants from islet cultures.....	98
Table 3.4: ELISA plate readings of bFGF concentration (pg/ml) in supernatants	99
Table 3.5: Analysis of different RNA purification methods.....	102
Table 3.6: Analysis of RNA isolated from islets	102
Table 3.7: Primer pairs evaluated but not suitable for final experiments.	103
Figure 3.6: Gel electrophoresis of PCR products using primers.....	104
Figure 3.7: Reduction of genomic DNA contamination by DNase treatment of RNA.....	106
Figure 3.8: Real time PCR amplification of cDNA and RNA before and after DNase treatment.....	107
Figure 3.9: PCR amplification of genomic DNA and cDNA	108
Figure 3.10: PCR amplification of genomic DNA with different annealing temperatures, 57°C and 60 °C.	109
Figure 3.11: Electrophoresis gel of PCR products from amplification of RNA and cDNA using redesigned Ang1 and bFGF primers	111

Figure 3.12: Electrophoresis gel of PCR products from amplification of RNA template and cDNA template in the presence of 2% deionised formamide and using annealing temperature of 63.5°C	112
Figure 3.13: Relative change in GAPDH expression in control culture and desferrioxamine treated islets when compared to fresh islets	114
Figure 3.14: A histogram plot of FL1 (annexin V-FITC) against FL3 (propidium iodide) when analysing dual stained cells.	116
Figure 3.15: Flow cytometry histograms from experiments with PBMC.....	117
Figure 3.16: Flow cytometry histograms from experiments with dispersed islets.	118
Figure 3.17: Apoptosis assessment of dispersed islets using AV-FITC and 7AAD.....	119
Figure 3.18: Assessment of viability in whole islets.....	122
Figure 3.19: Gel electrophoresis of purified IMAGE clone sequences	124
Figure 3.20: DNase digestion of rat Y sequence	126
Table 3.8: Internal primers of the Y chromosome and their product length.....	127
Figure 3.21: Y probe amplification using different primers	127
Figure 3.22: Y chromosome FISH of female (F) and male (M) hepatic tissue	129
Chapter 4	135
Figure 4.1: Change in mean blood sugar over time in all female recipients of male islets via the right branch transplant method.....	137
Figure 4.2: Change in mean weight over time in all female recipients of male islets via the right branch transplant method.....	138
Figure 4.3: Low power view of native islets within a rat pancreas.....	140
Figure 4.4: High magnification view of small native islets within the rat pancreas.....	142
Figure 4.5: Average sized native islets within the rat pancreas.	143
Figure 4.6: Large sized native islets within the rat pancreas.	144
Figure 4.7: Islets on the day of transplantation	147
Figure 4.8: Islets one day after transplantation	149
Figure 4.9: Islets three days after transplantation.	151
Figure 4.10: Liver sections 3 days after transplantation showing probable unengrafted islets.	152
Figure 4.11: Islets 5 days post transplantation.....	154
Figure 4.12: Islets 7 days post transplantation.....	156
Figure 4.13: Islets fourteen days post transplantation.....	158
Figure 4.14: Islets thirty days post transplantation.	160
Figure 4.15: Quantitative changes in average islet area and percentage of islet comprised of endothelium during engraftment.....	163
Figure 4.16: Quantitative changes in islet vascular density during engraftment.	164
Figure 4.17: Branching index in native and engrafting islets.	165
Chapter 5	171
Figure 5.1: VEGF concentration in supernatants from islet cultures after overnight incubation in increasing concentrations of desferrioxamine.....	174
Figure 5.2: Corrected bFGF concentration from 350 lysed islets after overnight culture in increasing concentrations of DFO or lysed fresh islets.....	175
Figure 5.3: VEGF concentration in supernatants from extended islet cultures in M199 medium after overnight incubation in desferrioxamine.	176

Figure 5.4: The effect of desferrioxamine treatment on the expression of angiogenic growth factor RNA expression, compared to fresh islets.	177
Figure 5.5: The effect of desferrioxamine treatment on the expression of angiogenic growth factor RNA expression, compared to control cultured islets.	178
Figure 5.6: Viability of cells within islets assessed by direct visualisation or dispersion and flow cytometry.....	181
Figure 5.7: Assessment of apoptotic and viable cells from dispersed islets.	182
Table 5.1: Percentage viable cells in control and desferrioxamine treated groups when assessed by 7AAD in dispersed islets.	183
Figure 5.8: Insulin concentrations in supernatants from glucose stimulation tests performed on control and desferrioxamine treated islets.	184
Table 5.2: Stimulation indices from glucose stimulation tests performed on control and desferrioxamine treated islets.....	185
Figure 5.9: Mean insulin content per islet of control and desferrioxamine treated islets.	186
Chapter 6	204
Table 6.1: Change in body weights and blood sugar after transplantation of 350 desferrioxamine treated islets.....	207
Figure 6.1: Change in mean non-fasting blood glucose over time in diabetic recipients of 350 desferrioxamine treated islets.....	208
Table 6.2: Significances for the difference between transplanted groups in the change of blood glucose after transplantation	208
Figure 6.2: Change in mean body weight over time in diabetic recipients of 350 desferrioxamine treated islets.....	209
Table 6.3: Change in body weights and blood sugar concentrations of diabetic rats after transplantation with 550 desferrioxamine treated islets.....	211
Figure 6.3: Change in mean non-fasting blood glucose over time in diabetic recipients of 550 desferrioxamine treated islets.....	212
Figure 6.4: Change in mean body weight over time in diabetic recipients of 550 desferrioxamine treated islets.....	213
Figure 6.5: Dual stained islets from recipients of 350 fresh islets.	215
Figure 6.6: Dual stained islets from recipients of 350 control cultured islets.....	216
Figure 6.7: Dual stained islets from recipients of 350 DFO 100 islets.	217
Figure 6.8: Dual stained islets from recipients of 350 DFO 1000 islets.	218
Figure 6.9: Average islet area of control and desferrioxamine treated groups.	219
Figure 6.10: Vascular densities and percentage vascular area of transplanted control and desferrioxamine treated islets.....	221
Chapter 7	232
Figure 7.1: Average number of pancreas offers per month	235
Figure 7.2: Reasons for refusal of pancreas offers.....	235
Figure 7.3: Islet equivalent yield from pancreas preparations over time.	237
Figure 7.4: Schematic diagram of the cooling device for the Cobe 2991.....	240
Figure 7.5: Final prototype of the cooling device used for refrigerating the Cobe 2991 centrifuge.	241
Figure 7.6: Temperatures achieved in the digest and centrifuge bowl with and without the cooling device.	242

Figure 7.7: Oral ulceration in islet recipient 1	245
Figure 7.8: Selected parameters from recipient 1.	247
Figure 7.9: Selected parameters from recipient 2.	249
Figure 7.10: Selected parameters from recipient 3.	252
Chapter 8	258
Figure 8.1: Schematic insulin profiles in normal individuals, exogenous insulin administration and after islet transplantation.	282

Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine transaminase
Ang 1	Angiopoietin 1
Ang 2	Angiopoietin 2
AO	Acridine Orange
AST	Aspartate transaminase
AV	Annexin V
BS-1	Bandeiraea Simplicifolia
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CAD	COOH-terminal transactivation domain
CMRL	Connaugh Medical Research Laboratories, cell culture medium
CMV	Cytomegalovirus
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DFO	Desferrioxamine
DNA	Deoxyribonucleic acid
FCS	Foetal calf serum
FIH-1	Factor-inhibiting HIF 1
FISH	Fluorescent in-situ hybridisation
FITC	Fluoresceine isothiocyanate
GFP	Green fluorescent protein
HBSS	Hanks balanced salt solution
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HPH	Hypoxia-inducible factor prolyl hydroxylase
HSV	Herpes simplex virus
IEQ	Islet equivalents
ODD	Oxygen-dependent degradation site
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PI	Propidium iodide
RNA	Ribonucleic acid
RT	Room temperature (for centrifugation steps)
SPK	Simultaneous pancreas kidney transplant
STZ	Streptozotocin
TBS	Tris buffered saline
TE	Tris-EDTA
TMRE	Tetramethylrhodamine ethyl ester
UTR	Untranslated regions
UW	University of Wisconsin solution
VEGF	Vascular endothelial growth factor

Chapter 1 - Introduction

1.1 Impact of diabetes

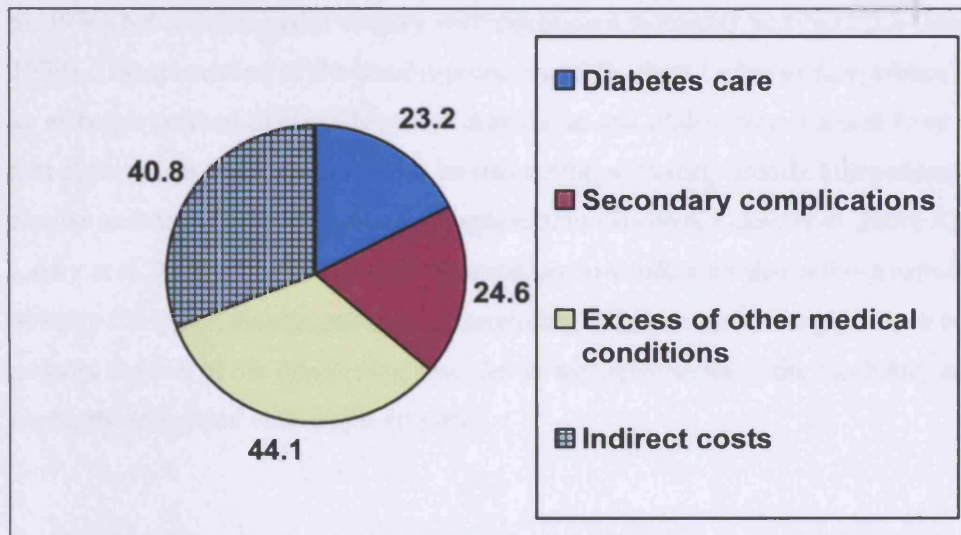
Diabetes mellitus is an epidemic of global proportions. The incidence and prevalence of this common disease is increasing and diabetes will become a major global health care issue over the next century. In 1995, diabetes mellitus affected 135 million people world wide and is due to double in prevalence by 2025 (King, Aubert et al. 1998). Although the major increase in prevalence will be due to type 2 diabetes, the global incidence of type 1 diabetes is also increasing by 3% per year (Onkamo, Vaananen et al. 1999). In the United Kingdom, diabetes affects 2.1 million people of which about 200,000 have type 1 diabetes. The incidence of type 1 diabetes in the United Kingdom is increasing by 1.9 to 9.5 % every year and by 2010 the incidence will have almost doubled (Onkamo, Vaananen et al. 1999).

The increasing incidence and prevalence will have major financial implications as health care services face the cost of treating diabetes and its associated complications. In 2002, the American Diabetes Association (ADA) estimated the total annual cost of diabetes in the US to be \$132 billion (see figure 1.1), of which \$92 billion was spent on direct healthcare costs and \$40 billion was lost due to disability (ADA 2003). The direct medical costs of diabetes in the United States represent 19% of total personal health care expenditures while diabetics only account for 4.2% of the US population (ADA 2003). In the United Kingdom, the overall healthcare cost of diabetes is unclear, although Evans and colleagues estimated that within their local population of Dundee, diabetic patients consumed 8% of the overall NHS drug budget while only accounting for 1.9% of the local population (Evans, MacDonald et al. 2000). A further study within the same population also estimated the rate of severe hypoglycaemia (requiring outside help) to be 12 per 100 patient years, at a local cost of over £92,000 per annum and estimated national cost of over £13 million (Leese, Wang et al. 2003).

As well as the impact on health expenditure, diabetes also reduces health-related quality of life (Hart, Bilo et al. 2003). The reduction in quality of life is not only associated with

complications of the disease, most particularly macrovascular complications (Hart, Bilo et al. 2003), but further decreases with increasing intensity of treatment (Maddigan, Majumdar et al. 2003). In order to improve the treatments for diabetes, a therapy would therefore have to reduce the secondary complications whilst improving quality of life and be cost effective.

Figure 1.1: Cost of diabetes in the United States (in billions of US dollars). Solid slices represent direct medical costs and crosshatched represent indirect costs. Statistics are from the American Diabetes Association, 2002.



1.2 Treatment options for diabetes

The current treatment for most patients with type 1 diabetes is insulin, with the aim of providing near normal glycaemic control. However, despite treatment with insulin, most patients do not achieve normal metabolic control of their diabetes and will still acquire secondary complications. Clinical trials indicate that improved control of blood glucose with intensive insulin regimes can reduce the incidence of secondary diabetic complications but dramatically increases the risk of potentially dangerous episodes of hypoglycaemia (DCCT 1993). Transplantation of the whole pancreas achieves optimal metabolic control without an increased risk of hypoglycaemia (Robertson, Sutherland et al. 1996) but requires major surgery with significant morbidity and mortality (Manske 1999). Transplantation of the insulin-producing cells alone (Islets of Langerhans) offers an effective method of obviating these drawbacks and studies from Canada have shown that clinical islet transplantation can be successful, with early insulin independence rates similar to those of whole pancreas transplantation (Shapiro, Lakey et al. 2000; Ryan, Lakey et al. 2002). Clinical islet transplantation now offers an alternative treatment strategy for type 1 diabetic patients by providing an improvement in glycaemic control without the risk of life threatening episodes of hypoglycaemia or the morbidity and mortality associated with major surgery.

1.3 Clinical islet transplantation

The first reported non vascularised pancreas transplant was performed in Bristol (UK) in 1894 by P. Watson Williams, who implanted sheep pancreatic tissue under the skin of a 15 year old diabetic boy. Unfortunately, the boy died 3 days later and only one further attempt was recorded, in 1924, until the successes of Najarian who introduced islets into the peritoneal cavity of a diabetic patient (Najarian, Sutherland et al. 1977).

Long term function of allografted islets was first reported in 1980 (Largiader, Kolb et al. 1980) but reproducible insulin independence was only achieved in the 1990s. At this time intra-portal islet transplantation, embolisation of islets into the liver via the portal vein, had become the recognised route for administering islets. Despite advances in isolation, purification and transplantation techniques, insulin independence rates were still poor with only 8-14% of patients free from insulin after one year (Islet Transplant Registry, Giessen). The Edmonton protocol (Shapiro, Lakey et al. 2000) improved the 1 year insulin independence rates to a more respectable level of 80% (Ryan, Lakey et al. 2002) and transformed islet transplantation from a research technique to a clinical tool for the treatment of diabetes.

Since the publication of the Edmonton paper (Shapiro, Lakey et al. 2000), a number of centres worldwide have set out to use this protocol and repeat the success of this original investigation. The best indicator of the reproducibility of the Edmonton protocol will probably be the Immune Tolerance Network trial. This trial has set out to examine the outcomes of patients receiving islet transplants under the Edmonton protocol within 9 centres throughout North America and Europe. Although the trial has not yet reported, interim data presented at the American Transplant Congress in 2004 suggests that the technique is transferable with 53% of patients becoming independent of insulin and a further 19% who are C peptide positive on reduced doses of insulin, after at least one year follow up (Shapiro, Ricordi et al. 2004). Unfortunately, a further 17% of patients had primary non function of their grafts and 11% withdrew due to complications. These results were disappointing when compared to the original Shapiro data and have been further compounded by poor long term graft survival which suggests that insulin

independence only lasts for a median of 15 months and less than 10% remain insulin independent after 5 years (Ryan, Paty et al. 2005). Despite the poor long term insulin independence rate, patients with a functioning graft (C peptide positive) had an improved hypoglycaemic score and metabolic function compared to patients with a failed graft; HbA1c of 6.7% in C peptide positive, insulin requiring patients compared with an HbA1c of 9.0% in C peptide negative recipients.

Islet transplantation has advanced a long way since the first xenogenic transplant in the late 19th century but progress has been slow. The early success of the Edmonton protocol has been tempered with poor long term graft survival but islet transplantation still remains a useful clinical tool and the number islet transplant centers around the world are still increasing.

1.4 Clinical islet transplantation in the United Kingdom

1.4.1 History and current situation of UK clinical islet transplantation

The first human allogenic non vascularised pancreas transplant in the UK was performed in 1916 at Newcastle-upon-Tyne by F.C. Pybus (Pybus 1924). In this case, human allogenic tissue was transplanted underneath the skin of the abdominal wall without immunosuppression and was rapidly destroyed. Despite a slight and temporary reduction of glucose concentrations in one recipient, the grafts “failed to relieve the diabetes”.

Intra portal allogenic islet transplantation, as we know it today, was not performed for another 66 years until Prof Nick London’s team in Leicester transplanted a patient on the 2nd of August 1991. This was followed by Prof Derek Gray and colleges in Oxford who performed another transplant on the 31st August 1991. This initial success was then followed by a further 10 allogenic islet transplants, 8 at Oxford and 2 at Leicester. The initial results from these patients were disappointing with C peptide becoming positive in only a few patients and no insulin independence achieved. With the improved results and safety of whole pancreas transplantation, the interest in islet transplantation in the UK diminished and the last transplant, under the original protocol, was performed at Oxford in 1998.

It was not until the excitement of the data from Edmonton that interest in human islet transplantation was rekindled and a number of centres started to re-establish islet transplant units within the UK. Since then, and as of January 2006, eight further patients have been transplanted in the UK using the Edmonton protocol (4 patients at Kings College hospital, 3 at the Royal Free hospital and 1 at the Churchill hospital in Oxford). In addition, three further centres (Bristol, Leicester and Edinburgh) are establishing islet isolation programmes with a view to allogenic islet transplantation.

1.4.2 Funding and staffing of UK islet transplant centres

Despite the renewed interest in clinical islet transplantation within the UK, the establishment of clinical programmes remains difficult and centralised public healthcare

funding is not forthcoming. The reason for the reluctance to acknowledge clinical islet transplantation as a treatment option in the UK is probably related to the high cost to benefit ratio and perceived lack of efficacy of islet transplantation within the UK. This view point has been reinforced by the latest National Institute of Clinical Evidence (NICE) guidelines for Pancreatic Islet Cell Transplantation (Appendix 2), which have not endorsed the technique as an accepted treatment within the UK.

"Current evidence on the safety and efficacy of pancreatic islet cell transplantation does not appear adequate to support the use of this procedure without special arrangements for consent and for audit or research."

NICE guidance issued October 2003.

This difficult funding situation has been further compounded by the issue of whether islet transplantation should be considered as research or an accepted clinical treatment. Therefore, public healthcare funding is not available because national recommendations suggest the technique is still a research tool and research funding bodies have accepted the published data from international centres and classify islet transplantation as an established clinical treatment. The poor level of funding available is further added to by the high standards of manufacturing (set by the Medicines and Health Related Agency) required of an islet isolating facility and the number of staff required to run an isolation unit on a continuous basis and comply with European Working Time Directive.

The current situation in the United Kingdom has meant that islet isolation facilities are funded by charities which support the treatment of diabetic patients and staff are funded by research grants in areas allied to islet transplantation. The cost of transplanting patients with islets is currently funded by Diabetes UK but this will only stretch to the first 15 transplants and it is unclear where funding will come from in the future.

1.4.3 Isolation and purification of human islets for transplantation

After funding and staffing issues have been addressed, the process of islet isolation has to be considered. Human islet isolation is a complicated and lengthy procedure which starts with the provision of a healthy and suitably retrieved pancreas. Islets are then

digested from the exocrine pancreas using a blend of collagenase and neutral protease enzyme and mechanical dissociation. The resultant islet/exocrine mixture is subsequently purified using a Ficol based gradient loaded onto a Cobe 2991 cell separator (Gambro BCT, Germany). Semi-pure islet preparations are either cultured for up to 3 days in CMRL (Connaugh Medical Research Laboratories, cell culture medium) based medium or transplanted fresh into the portal vein via a radiological approach.

Although the procedure of islet isolation is based on the semi-automated technique originally described by Ricordi (Ricordi, Lacy et al. 1988), the technique is by no means standardized and there are multiple factors which influence the outcome of the procedure. The complicated and variable isolation technique gives rise to a steep learning curve for centres setting up an islet isolation facility which only compounds the cost and staffing issues. Once consistent islet isolation is established, there are further hurdles to overcome, such as sterile handling, product release, infusion, patient listing and patient management.

1.4.4 Donor pancreases for islet isolation

The isolation of islets for transplantation is dependent on a supply of donor pancreases, with well procured organs leading to improved islet isolation (Nano, Clissi et al. 2005) and transplant outcome (Goto, Rahman et al. 2005). The general shortage of organ donors means that there are few donor pancreases available for transplantation and current practice in the UK gives priority of organ placement to whole organ transplantation. In addition, only 27% of potentially suitable donors have the pancreas retrieved, in spite of a further 12% being offered but not retrieved (Ridgway, White et al. 2005). The small quantity of donor pancreases received at islet isolation centres prohibits the necessary number of processes required to maintain the basic skills for islet isolation and a better supply of quality organs will be essential to the maintenance of clinical islet transplant programmes in the UK.

1.4.5 Patient selection and management

The current indications for islet transplantation in type 1 patients are hypoglycaemic unawareness, severe progressive secondary complications, despite best intensive insulin treatment, and patients who are already immunosuppressed for another transplant (Federlin and Pozza 1999; Bertuzzi, Secchi et al. 2004). Despite these broad categories, there are surprisingly few patients who are truly suitable for islet transplantation and French studies would suggest that only 27% of islet alone patients referred by their diabetologists to a transplant centre are suitable for assessment (Vantyghem, Hazzan et al. 2004). This situation is further compounded by the lack of data for the risk benefit ratio of islet transplantation.

Although improved glycaemic control in type 1 diabetics has been shown to reduce secondary diabetic complications (DCCT 1993), a reduction in mortality from improving diabetic control is less clear. Extrapolations can be made from reducing known mortality risk factors, such as nephropathy (Go, Chertow et al. 2004), or observational studies of lower mortality associated with lower baseline glycated haemoglobins in type 1 (Klein 1995) and type 2 diabetics (Stratton, Adler et al. 2000). It is also possible that the abolition of hypoglycaemic unawareness by transplanting patients with islets may reduce mortality but only a small number of patients have been transplanted so far and there is no comparative data to prove any benefit. The situation where the argument for reduced mortality from better glycaemic control is probably stronger is in patients who receive renal transplants. In this situation, beta cell replacement in the form of a whole pancreas at the time of renal transplantation has been shown to reduce mortality when compared to kidney transplant alone (La Rocca, Fiorina et al. 2000; Reddy, Stablein et al. 2003). The further benefit of islet transplantation over pancreas transplantation is the reduced morbidity and mortality. Although no formal assessment of risk of death from islet transplantation has been performed, there has not been a single death directly related islet transplantation so far. This compares favourably to whole organ transplantation, where the relative risk of death is increased between 2.4 to 6.1 times within the first 90 days post transplantation, depending on whether the

pancreas is transplanted with or without a kidney respectively (Venstrom, McBride et al. 2003; Gruessner, Sutherland et al. 2004).

Any benefit of islet transplantation, either perceived or proven, has to be balanced against the risks of transplantation. Although there are some peri-procedural risks from islet transplantation (such as a 4% risk of reversible portal vein thrombosis and roughly 10% risk of hepatic bleeding), the main risks are associated with immunosuppression. Once again, there is little islet specific literature but if an extrapolation is drawn from solid organ transplantation, the main risk comes from infection, malignancy and direct toxic effects of immunosuppressants. Infection occurs in 15-44% of renal transplant recipients within the first year (Soulillou and Giral 2001) and accounts for 15-20% of deaths following renal transplantation (Briggs 2001). In addition, the risk of malignancy is increased in transplant recipients and a study from the United Kingdom has estimated that the risk of malignancy 20 years post transplant was 40% compared with a 6% cumulative risk in an age matched control population (London, Farmery et al. 1995). This increased risk is further backed up by registry data in the United States which estimates a 3-4 times increased relative risk for all malignancies in transplant patients, with a range from 2 times relative risk for colonic cancer and up to 500 times relative risk of Kaposi's sarcoma (Penn 2000). Lastly, the immunosuppressants have important side effects which include dyslipidaemia, lymphopenia, mouth ulcers, diabetes and most importantly, nephrotoxicity.

All the risks and benefits have to be considered and explained to potential recipients before listing a patient for islet transplantation. At present, our experience with islet transplantation is limited and clinicians must err on the side of caution when considering patients for transplantation. With time, we will gain experience and a better understanding of where this potentially promising treatment fits into the array of therapies available to diabetic patients. In the meantime, we must fully inform potential recipients of the poor long term insulin independence rates achieved with clinical islet transplantation and look to ways of improving clinical outcome and reducing the number of donors required to induce insulin independence.

1.5 Insulin independence and peri-transplant islet loss

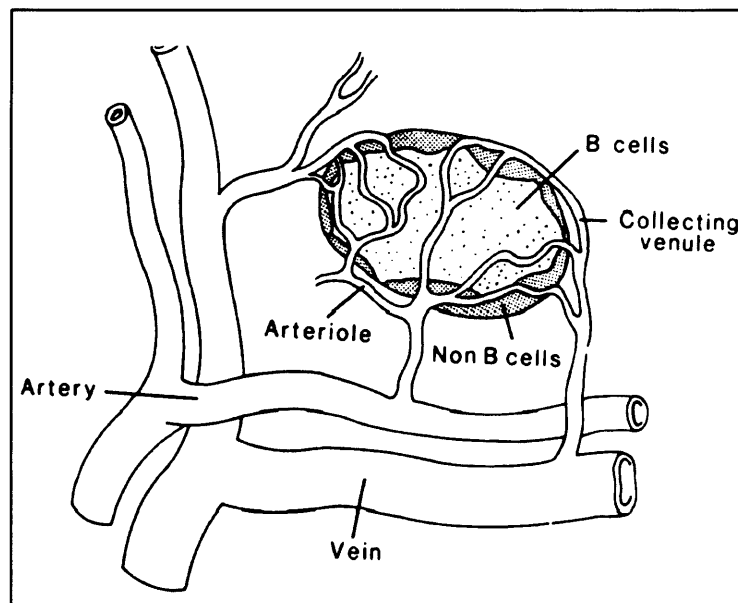
The original publication by Shapiro and his colleagues (Shapiro, Lakey et al. 2000) showed that acceptable rates of early insulin independence were achievable. The impressive levels of early insulin independence achieved by the Edmonton protocol was probably due to a number of changes made to the isolation and transplant protocol, including use of non-xenogenic proteins, a new steroid free immunosuppressive regime, transplantation of freshly isolated islets, rapid sequence transplantations and most importantly, use of a large dose of fresh islets (~11,000 islet equivalents per patient) (Shapiro, Lakey et al. 2000). In order to achieve this high dose of islets, each recipient required islets from at least 2 donor pancreases.

This need for multiple donors contrasts with the single donor to recipient required for insulin independence after whole pancreas transplantation and emphasises that successful islet transplantation is dependent on the number of islets transplanted and perhaps, more critically, the proportion which survive. Indeed, up to 60% of donor islets may be lost in the immediate post transplant period due to necrosis and apoptosis (Biarnes, Montolio et al. 2002). The cause for this high rate of loss remains unclear but is probably related to the disruption of the normal islet microvasculature during isolation from the whole pancreas and the hypoxic environment of the portal vein, into which the islets are transplanted. In order to investigate this reasoning, the native and transplanted islet vasculature should be considered further.

1.6 Native islet structure and vasculature (Figure 1.2)

Pancreatic islets are organised conglomerations of different cells which produce a variety of endocrine hormones. The structure of islets consist of a central core of β cells, which produce insulin, surrounded by an outer mantle of α , δ and pp cells which secrete glucagon, somatostatin and pancreatic polypeptide, respectively. In the rat, the sizes of the islets are variable and range from 30 to 300 μ m (Lifson, Lassa et al. 1985). Each islet is highly vascular and although islets only account for 0.3% of the volume of the pancreas, they receive 6% of the arterial blood flow with smaller islets receiving relatively greater blood flow per volume than larger islets (Lifson, Lassa et al. 1985). Each islet is supplied by one to three afferent arterioles which divide at the mantle of the islet into a glomerular-like network of capillaries and either coalesce at the edge of the islet to form a network of collecting venules or traverse the central beta core of the islet to connect with the collecting venules on the other side (Bonner-Weir and Orci 1982; Liu, Guth et al. 1993). The venules then either drain into the acinar (exocrine) venous system or directly into interlobular veins and subsequently into the portal vein (Liu, Guth et al. 1993).

Figure 1.2: Structure and vascular supply of native islets, modified from (Bonner-Weir and Orci 1982).



1.7 Transplanted islet vasculature

A number of animal models (intra-portal, kidney subcapsular and skin fold) have been used to investigate the revascularisation of transplanted islets and will be discussed later in section 1.12.

1.7.1 Structure of transplanted islet vasculature

Studies on experimental animals show that transplanted islets revascularise and reform a glomerular-like network of blood vessels (Vajkoczy and Menger 1995). The process of revascularisation can take up to 14 days (Vajkoczy, Menger et al. 1995) and further dynamic reorganization of blood vessels may continue after a month post transplant (Rooth, Dawidson et al. 1989). During this period of time the islets remain hypoxic and, even after the formation of new vessels, the partial pressure of oxygen and vascular density are reduced when compared to endogenous islets (Carlsson, Palm et al. 2002). The reduction in oxygen partial pressures and vascular density persists and can still be seen 6 months post transplant (Jansson and Carlsson 2002; Mattsson, Jansson et al. 2003). These observations have led to the suggestion that hypoxia and poor revascularisation are the main factors responsible for the failure of transplanted islets in the immediate post-transplant period (Carlsson, Palm et al. 2002).

Intra-portal transplanted islets revascularise from the hepatic arterial system with venous drainage into the portal vein (Griffith, Scharp et al. 1977; Andersson, Korsgren et al. 1989). The revascularisation of intra-portal islets begins around day four post transplantation and is usually completed between days eight and eleven (Griffith, Scharp et al. 1977).

1.7.2 Origin of transplanted islet endothelium

The origin of the endothelium within transplanted islets is unclear but would be expected to have an impact on both allorecognition and immune destruction of a transplanted graft. Intravital microscopy studies of islets transplanted into the dorsal skin fold of nude mice have suggested that endothelium grows into the islet from the host arterial system (Vajkoczy, Menger et al. 1995), suggesting that transplanted

islets gain their endothelium from a host source. The authors further substantiated this observation by using a xenogenic dorsal skin fold model of islet transplantation to track the origin of the endothelium of transplanted islets (Vajkoczy and Menger 1995). Species-specific antibodies for endothelium (anti-PECAM-1 and anti-factor VIII) were used to delineate the origin of the endothelial cells and suggested that the new endothelium was derived entirely from the recipient. This model gives some insight into the origin of the endothelium but is compromised by the site to which the islets are transplanted, the endothelial markers chosen for the experiment and the cross species model used. It is possible that the antibodies may have cross reacted in this experiment and the endothelial markers used may not have been as robust as the lectin *Bandeiraea Simplicifolia* (Mattsson, Carlsson et al. 2002). In addition, the authors did not mention any complications of cross species transplantation which may have interfered with the results. A further study by Linn and colleagues has subsequently shown that free islets embedded in a fibrin matrix develop outgrowths of endothelial cords from the islet into the fibrin matrix (Linn, Schneider et al. 2003). When they then used a Lac-Z reporter gene in islets transplanted into wild type mice, donor endothelium was found around and within the transplanted islet, suggesting that donor endothelium may also play a role in the revascularisation of transplanted islets.

1.8 Control of the revascularisation process

The factors which control the revascularisation process have yet to be fully elucidated, although there is some evidence from less clinically relevant models of islet transplantation. In-vitro studies of cultured islets have shown a 3 fold increase in the mRNA expression of the angiogenic polypeptide vascular endothelial growth factor (VEGF) after 2 days in culture (Vasir, Aiello et al. 1998). In kidney sub-capsular models of islet transplantation, the angiogenic factors basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF β) and VEGF are found in increased concentrations during the early stages of the revascularisation process (Vasir, Reitz et al. 2000). These molecules are known to be increased in response to hypoxia and can modulate the neovascularisation process, thus making them likely candidates to control revascularisation. VEGF is probably a major factor in revascularisation but its expression is only increased for the first two to three days in transplanted islets and levels return to normal by day 5 post transplant (Vasir, Jonas et al. 2001). This pattern of expression would suggest, as in revascularisation of other tissues, that VEGF initiates revascularisation but other factors are needed for the formation of a mature network of vessels (Yancopoulos, Davis et al. 2000). In models of angiogenesis, over-expression of VEGF alone leads to the formation of large numbers of leaky vessels whereas over-expression of both VEGF and angiopoietin 1 (Ang-1) leads to the formation of an increased number of larger, leakage resistant vessels (Thurston, Suri et al. 1999; Thurston, Rudge et al. 2000).

The angiopoietin family of endothelial specific growth factors has recently been characterised and consists of 4 molecules (Ang 1, Ang 2, Ang 3 and Ang 4) which exert their action mainly through the Tie 2 receptor. Ang 1 and Ang 2 are the better characterised molecules and seem to work in combination with VEGF for optimal blood vessel formation. In contrast to VEGF, Ang 1 does not induce endothelial cell proliferation but seems to mediate the interaction between the endothelial cells and surrounding matrix and support cells (Suri, Jones et al. 1996). Ang 2 is a natural antagonist to Ang 1 with similar affinity for Tie 2 receptors and probably blocks the stabilising effects of Ang 1 on vessels, creating destabilised vessels which are prone

to regression. Therefore, Ang 2 would have more of a role in remodelling of vessels by allowing vascular beds to be more flexible (Maisonpierre, Suri et al. 1997). In corpus luteum development, Ang 2 is expressed in the early stages together with VEGF and is thought to block the stabilisation of vessels allowing them to remain more responsive to the proliferative and sprouting effects of VEGF (Maisonpierre, Suri et al. 1997). In contrast, Ang 1 expression is found later in corpus luteum development and is associated with stabilisation of the vessels. It is therefore possible that Ang 2 may be expressed in the very early stages of islet revascularisation, when there is known to be regression of endothelium and lack of capillary spaces (Griffith, Scharp et al. 1977). The regression process would then be followed by new vessel formation, under the influence of VEGF and other factors, and a subsequent maturation process under the influence of Ang 1.

1.9 Manipulation of the revascularisation process

Our present understanding of the molecular mechanisms necessary for the revascularisation of transplanted islets has allowed an insight into how this process could be manipulated. With this knowledge, the aim would be to target the mechanisms responsible in order to accelerate the revascularisation of transplanted islets and hopefully reduce the beta cell loss. A number of authors have investigated methods for increasing the expression of single growth factors either by local delivery of the molecule (Nakano, Yasunami et al. 2000) or adenoviral-mediated gene delivery (Garcia-Ocana, Takane et al. 2003; Lopez-Talavera, Garcia-Ocana et al. 2004; Zhang, Richter et al. 2004).

Increased delivery of hepatocyte growth factor (HGF) to islets by adenoviral techniques has been shown to reduce beta cell death, improve graft function, increase the insulin content and number of islets surviving intra-portal transplant and hence augment the reversal of diabetes by marginal islet transplants (Garcia-Ocana, Takane et al. 2003; Lopez-Talavera, Garcia-Ocana et al. 2004). A further study by Zhang and his colleagues increased the expression of VEGF by adenoviral techniques and found an improvement in the reversal of diabetes and vascular density in treated islets transplanted under the renal capsule of mice (Zhang, Richter et al. 2004). Although these experiments provide a good insight into the manipulation of the revascularisation process, they rely on the insertion of new genetic material into the cells and use viral transfection systems which may increase the immunogenicity of transplanted cells. In addition, the percentage of cells over-expressing the target factor can be as low as 30% (Garcia-Ocana, Takane et al. 2003) and over-expression of only a single target molecule is achieved, rather than inducing a more coordinated and physiological response involving several growth factors.

1.10 Hypoxia sensing and hypoxia inducible factor

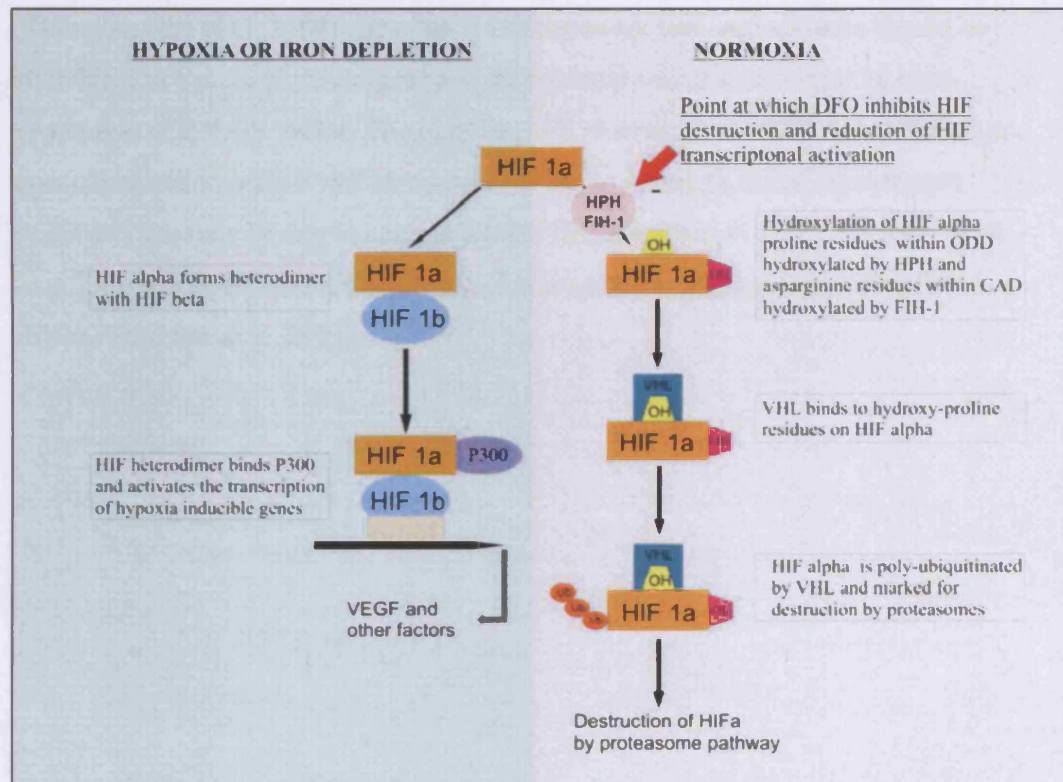
The expression of some angiogenic factors are controlled by the hypoxia sensing pathway (Wenger 2000) and may therefore be amenable to manipulation at a higher point in the hypoxia activation cascade. The benefit of this approach is to produce a more balanced and physiological approach to accelerating revascularisation which should provide all the angiogenic factors necessary for the formation of a mature and intact vasculature. The mechanism responsible for sensing hypoxia (figure 1.3) is dependent on hypoxia-inducible factor (HIF), and has recently been reviewed (Wenger 2000; Lando, Gorman et al. 2003). In hypoxic conditions, the heterodimeric HIF-1 transcription factor is formed by the combination a HIF alpha (HIF α) subunit binding to a HIF beta subunit. This transcription factor can directly bind to DNA in hypoxia responsive elements of oxygen-regulated genes, in order to alter the expression of these molecules.

In normoxic conditions, HIF α is metabolised in order to regulate the expression of hypoxia response genes. The first step in the metabolism of HIF α is by the iron and oxygen dependent group of enzymes, HIF prolyl hydroxylases (HPH) (Jaakkola, Mole et al. 2001; Yu, White et al. 2001). These enzymes hydroxylate proline residues within the oxygen-dependent degradation site (ODD) of HIF α and allow binding of the von Hippel-Lindau protein to HIF α (Maxwell, Wiesener et al. 1999). The von Hippel-Lindau protein poly ubiquitinates HIF α , which subsequently marks the molecule for destruction by proteosomes (Maxwell, Wiesener et al. 1999). HIF expression can also be regulated by the oxygen dependent hydroxylation of an asparaginyl residue in the COOH-terminal transactivation domain (CAD) of HIF α (Lando, Peet et al. 2002; Maxwell 2003). This hydroxylation limits the binding of the CAD with the p300 co-activator necessary for transcriptional activation, therefore regulating the activity of HIF-1. The hydroxylation of asparagine within the CAD is performed by the iron containing enzyme FIH-1 (factor-inhibiting HIF) which can be inhibited by iron chelators (Lando, Peet et al. 2002) and thus provides a further mechanism for the increased expression of HIF by agents such as DFO.

Figure 1.3: The metabolism of Hypoxia Inducible Factor alpha (HIF a) during normoxia, hypoxia and iron depletion.

Desferrioxamine (DFO) inhibits the iron dependent HIF prolyl hydroxylases (HPH) to reduce to destruction of HIF 1a and inhibits factor-inhibiting HIF (FIH-1) to reduce the blocking HIF transactivation. Abbreviations: HIF b = Hypoxia Inducible Factor beta, OH = hydroxyl group, VHL = Von Hippel Lindau protein, Ub = Ubiquitin residue, ODD = oxygen-dependent degradation site, CAD = COOH-terminal transactivation domain, P300 = CBP/P300 transcription adapter.

Modified from (George and Kaelin 2003) and (Maxwell 2003).



Hypoxia-inducible factor is known to positively regulate the expression of VEGF, VEGF receptor, Ang 2, Ang 4, Tie 2 and bFGF (Grimm, Wenzel et al. 2002; Yamakawa, Liu et al. 2003). Therefore, increasing the expression of HIF should subsequently up regulate these important angiogenic factors and provide a more coordinated signal to stimulate angiogenesis. Studies of transgenic mice over expressing HIF-1 α in the epidermis show an up regulation of VEGF mRNA by 8 to 13 times when compared to non transgenic animals (Elson, Thurston et al. 2001). In addition, the dermal blood vessels were increased by 30% but did not have increased vascular permeability and were of normal morphology, when compared to non transgenic animals (Elson, Thurston et al. 2001). This observation contrasts with the increased vascular permeability induced in animals over-expressing VEGF alone (Thurston, Suri et al. 1999) and adds to the argument that angiogenesis should be stimulated in a more physiological and coordinated way than the isolated over-expression of a single factor. The question still remains as to which other factors are over expressed to reduce vascular permeability, as although initial experiments suggested this may be due to angiopoietins (Thurston, Suri et al. 1999), Ang 1 and Ang 2 are not found at increased levels in transgenic mice over expressing HIF 1 α (Elson, Thurston et al. 2001).

1.11 Hypoxia sensing and desferrioxamine

The first step in the metabolism of HIF-1 α requires the iron dependent enzyme HPH (Jaakkola, Mole et al. 2001). Iron chelating agents, such as desferrioxamine (DFO), have been used to inhibit HPH with the effect of increasing the expression of HIF and the hypoxia regulated molecules VEGF, Tie 2 and angiopoietins (Yamakawa, Liu et al. 2003). Treating islets with desferrioxamine may provide a theoretically attractive technique for pre-treating islets before transplantation that does not require the insertion of new genetic material into the islets and should affect all transplanted cells. This method should enable the increased expression of a number of angiogenic factors post islet transplantation in a more physiological and coordinated fashion and hopefully improve islet engraftment.

Desferrioxamine and its subsequent up regulation of HIF-1 and erythropoietin have also been explored in other models of hypoxia, in order to precondition or salvage tissue from hypoxic damage. In a rodent model of cerebral infarction, desferrioxamine pre-treatment in-vivo was shown to reduce cerebral infarct volumes by up to 35% when compared to non desferrioxamine treated animals (Prass, Ruscher et al. 2002). Subsequent experiments on rat primary cortical neurons exposed to hypoxia and glucose deprivation also showed that pre-treatment with desferrioxamine in-vitro reduced cell death when compared to controls, with significant reduction occurring at 150 μ M desferrioxamine (Prass, Ruscher et al. 2002). A further study in an in-vivo rodent model of cerebral ischaemia looked at whether desferrioxamine treatment after a hypoxic insult could ameliorate cell damage. Desferrioxamine injected intra-peritoneally after a 1.5 hour ischaemic insult resulted in a significant reduction of cerebral volume loss and caused a significant up regulation of HIF-1 compared to control which extended after 24 hours post dose (Mu, Chang et al. 2005).

The mechanism by which DFO protects against ischaemic injury has not been fully elucidated. Some authors have suggested that protection in a neuronal ischaemic model may be due to the up regulation HIF-1 and subsequent increase of down

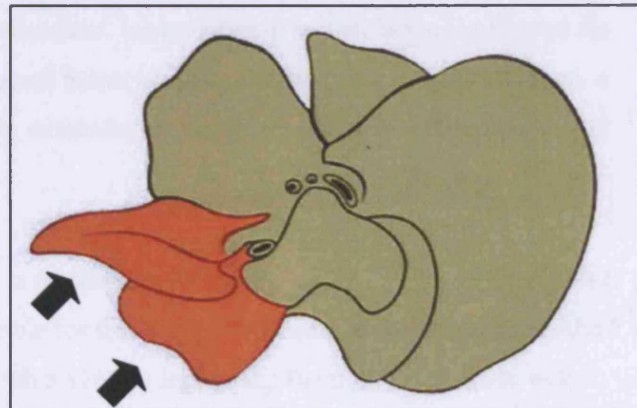
steam hypoxic response elements, such as erythropoietin (Prass, Ruscher et al. 2002; Mu, Chang et al. 2005). This theory has been substantiated by a reduction of desferrioxamine induced neuroprotection by HIF-1 α specific antisense oligonucleotides (Hamrick, McQuillen et al. 2005), although the neuroprotective effect of DFO was not fully abrogated. The incomplete blockade of neuroprotection by impeding the HIF pathway suggests that there may be another mechanism by which DFO affords protection against hypoxia. Iron chelators are known to interfere with Fenton chemistry (Merkofer, Kissner et al. 2006), the process by which oxygen radicals are formed, and DFO has been shown to reduce the formation of oxygen radicals (Kozlov, Ostrachovitch et al. 1994; Urbanski and Beresewicz 2000). Desferrioxamine is able to reduce the damage to cells caused by free radical formation (Horackova, Ponka et al. 2000; Yun, Takagi et al. 2003; Kurz, Gustafsson et al. 2006) and experiments in a hepatic ischaemia reperfusion model have shown that pre-treatment with DFO reduces secondary oxidative stress in the kidney (Polat, Tokyol et al. 2006). These experiments propose that DFO may reduce the cellular damage associated with oxidative stress and provide another mechanism by which DFO might protect islets from the insult of transplantation.

These experiments suggest that desferrioxamine may, in addition to up regulating VEGF, be able to “precondition” islets prior to transplantation with a view to reducing β -cell death after transplantation, thereby improving transplant outcome.

1.12 Experimental models of islet transplantation

The models of islet transplantation from which most results have been derived (kidney subcapsular or dorsal skin fold transplantation sites) were developed to permit visualisation of grafts and allow easy retrieval of the transplanted islets. The problem with these models is that the tissue into which the islets are transplanted may have different characteristics and responses to vascular growth factors than the more clinically relevant hepatic portal system. In addition, the process of injecting islets under the renal capsule or dorsal skin fold does not allow the assessment of an islet surrounded by host tissue as islets are often clumped in close association with each other. The clumping of islets and surrounding fluid may increase the diffusion distance for molecules, such as oxygen, increasing hypoxia and therefore altering revascularisation and expression of angiogenic factors. The drawback to intra-hepatic models has been the poor retrieval of islets which are widely dispersed throughout the liver. In order to combat this problem, our laboratory has validated an intra-portal transplant model for the localisation of islets to only 2 out of 7 lobes of the rat liver (20% of the liver volume, see figure 1.4). This method allows much better visualisation of islets and a greater number of islets per tissue slice (Juszczak M 2003).

Figure 1.4: Caudal view of rat liver showing the 2 lobes of the liver (arrowed) supplied by the right branch of the portal vein.



1.13 Purpose of research

Diabetes is a chronic disease with a major impact on both patient quality of life and health care costs. The current therapies for patients with type 1 diabetes do not provide the immaculate metabolic control necessary to stop or reverse the secondary complications of diabetes. Whole organ pancreatic transplantation can provide this level of metabolic control but requires a major abdominal operation which is associated with significant morbidity and mortality (Manske 1999) and is usually reserved for patients who are receiving a concomitant renal transplant. Islet transplantation offers an alternative therapeutic option to whole organ transplantation that is not associated with a high morbidity and mortality and is open to a wider range of patients (Federlin and Pozza 1999). The current limitation of islet transplantation is the need for two or more transplants per recipient in order to achieve insulin independence. The requirement for two or more transplanted organs is further compounded by the fact that only 50% of processed pancreases yield sufficient islets for transplantation so that each recipient may require four or more donor organs to achieve insulin independence. If a comparison is drawn from the Swiss-French experience, an average of 5.6 pancreas processes were required for each recipient at a cost of €5902 per process (including transport and procurement) (Guignard, Oberholzer et al. 2004). The overall cost for a single recipient was €77,745, of which pancreas procurement, transportation and isolation accounted for 43% of the total cost. If patients required fewer islets to achieve insulin independence, a smaller number of pancreas processes would be required and islet transplantation would become more cost effective.

The research described in this thesis is focused on the revascularisation of islets in order to elucidate the mechanisms responsible for this complex process and establish a method for manipulating revascularisation with a view to increasing the number of islets which survive after transplantation. If successful, the number of transplanted islets and donor organs required to achieve insulin independence could be reduced and more patients would be able to be transplanted from the limited resources and donor organs available.

Despite the current limitations, clinical islet transplantation has been accepted as a possible alternative to whole pancreas transplantation in patients who do not require renal replacement. The United Kingdom has been slow to initiate clinical islet transplant programmes and this thesis will explore the establishment of a clinical transplant programme and the methods required for optimising islet isolation and transplantation.

1.14 Hypothesis

1. Intra-portally transplanted islets revascularise in a coordinated and quantifiable fashion
2. Transplanted islet endothelium is derived from both donor and recipient.
3. Desferrioxamine treatment increases the expression of a number of key angiogenic factors required for the revascularisation of islets.
4. Increasing the expression of these key angiogenic factors early in the post transplant period will accelerate revascularisation of the islet, therefore reducing beta cell loss and improving post transplant outcomes.
5. Human clinical islet transplantation can be established in the United Kingdom

1.15 Aims of the research described in this thesis

1. Devise a robust methodology which will allow the clear examination and quantification of the revascularisation of intra-portally transplanted islets
2. Delineate the origin of transplanted islet endothelium.
3. Explore whether desferrioxamine treatment of islets improves revascularisation and post transplant outcomes.
4. Establish human islet isolation and transplantation at a centre within the United Kingdom.

Chapter 2 - Materials and methods

2.1 Commonly used solutions

Shown in Appendix 1

2.2 Common in-vivo techniques

The exploration of islet transplantation requires an in-vivo islet transplant model. We used a syngeneic transplant model between inbred Lewis rats. The in-vitro islet experiments were performed on islets isolated from locally bred Sprague Dawley rats.

2.2.1 Experimental subjects

Adult rats were used as donors of islets and recipients of islet transplants. Adult male Sprague Dawley rats (local source) of 250-300g were used as donors for in-vitro islet experiments. Inbred adult male Lewis rats (Harlan, Indiana, USA) of 250-300g were used as islet donors for in-vivo transplant experiments. Inbred adult male Lewis rats (Harlan) of 250-300g or inbred adult female Lewis (Harlan) rats 180-220g were used for syngeneic in-vivo transplant experiments. All animals were kept in accordance with Home Office regulations with free access to standard rat chow and water and 12 hour light/dark cycles.

2.2.2 Isolation of rat islets

Rats were anaesthetised with 24mg (0.4ml) of pentobarbitone (Rhone Merieux, France) intra-peritoneal (ip). After deep anaesthesia was achieved, a midline abdominal skin incision was made from xiphisternum to the pubic region. The abdominal cavity was opened and bowel retracted to expose the pancreas and duodenum. The proximal end of the common bile duct was isolated and a loose ligature passed around the duct. The distal end of the common bile duct, just before the insertion into the duodenum, was dissected free from the surrounding pancreas and a loose ligature passed around the duct. The duct was incised and a 2F cannula inserted into the duct toward the liver. Correct

positioning of the cannula was identified by the ante grade flow of bile down the cannula. The cannula was held in place by tightening the ligature around the distal end of the bile duct and the ligature at the proximal end was tightened to avoid retrograde flow of collagenase solution into the liver. The animal was exsanguinated through a jugular incision, the blood centrifuged and serum saved for future use in transplant solution. 3.3ml of ice-cold collagenase solution was injected down the cannula into the pancreas while the pancreas was directly observed to confirm uniform distension. The cannula was then removed and the pancreas completely excised.

The excised pancreas was briefly bench dissected to remove the spleen and associated fat and subsequently placed in a 30ml universal tube containing 10ml of HBSS at 37°C. The pancreas was incubated in the universal tube for 15-17 minutes (depending on activity of enzyme lot) at 37°C, aspirated twice through a 10G hollow needle and washed three times at 400 x g for 2 minutes room temperature (RT) in ice-cold HBSS supplemented with 0.5% BSA (HBSS/BSA). After the final wash, the pancreatic tissue was resuspended in HBSS/BSA and separated from any undigested lumps of pancreas. The digest was re-pelleted and bottom loaded onto Ficoll of density 1.084 (Histopaque, Sigma Chemicals, UK). Ice cold HBSS/BSA was layered on top of the Ficoll and the tube was centrifuged for 18 minutes at 600 x g at 4°C. After centrifugation, the pancreatic islets were harvested from the interface, resuspended in ice cold HBSS/BSA and washed a further 3 times before being transferred to a petri dish containing HBSS/BSA. Islets were then hand picked under an inverted stereo microscope and transferred to a 15ml polypropylene test tube.

2.2.3 Measurement of blood glucose

Capillary blood glucose was measured from the tails of rats, in order to reduce the stress and trauma of repeated tail tipping. The tails of rats were pricked with a 25G needle and massaged to express capillary blood. Blood glucose concentrations were measured with an Accu-Check Advantage II glucometer (Roche Diagnostics, Germany).

2.2.4 Induction of diabetes

Streptozotocin (STZ) was reconstituted in normal saline immediately before use at a concentration of 50 or 55mg/ml. Healthy adult male Lewis rats were weighed and injected i.p. with 55mg/Kg streptozotocin. Females received a dose of 50mg/Kg of streptozotocin, as higher doses had proved to induce fatal diabetic coma; probably due to a higher fat to lean body mass ratio in females. Animals were fed normal rat chow with free access to water. After 3 days, the animals were reweighed and blood glucose was estimated to confirm diabetes. Animals were considered to be diabetic and suitable for transplantation if blood glucose concentrations were over 20 mmol/l and animals had lost over 5% of body mass when compared to pre-streptozotocin levels.

2.2.5 Transplantation of islets

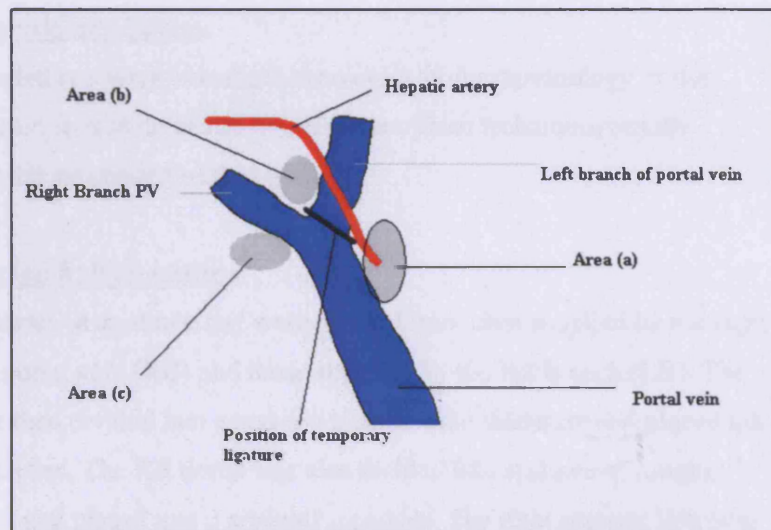
Transplant recipients were weighed and blood glucose was estimated, as above. Anaesthesia was induced with a 4% halothane in oxygen mixture in a small animal anaesthetic box. When anaesthetised, the abdominal area was shaved and the rat transferred to a warming board. Anaesthesia was maintained with a 1 – 1.5% halothane in oxygen mixture and the rats checked for level of anaesthesia by the hind paw withdrawal method. Rats were continuously checked throughout the operation for respiratory rate and depth of anaesthesia, with appropriate adjustment of halothane dose. Neuromuscular blocking agents were not used during the procedure.

When the animal was fully anaesthetised, the abdominal skin was washed with betadine solution and incised from xiphisternum to pelvic region. The abdominal cavity was opened, the rib cage gently retracted supero-laterally on either side and the bowel presented outside of the abdominal cavity onto gauze moistened with normal saline. The bowel was gently moved laterally to the left of the rat, in order to expose the porta hepatis, and covered with further saline moistened gauze. A dissecting microscope was employed to visualise the porta hepatis and assist transplantation (figure 2.1). The left aspect of the portal vein was dissected free just inferior to the bifurcation of the portal vein (area a, figure 2.1) and a dissecting forceps passed underneath the portal vein to an area just inferior to the bifurcation of the portal vein on the right side of the rat (area c,

figure 2.1). The posterior aspect of the portal vein was then dissected free from the surrounding fascia. Next, an area in between the two branches of the portal vein was dissected (area b, figure 2.1), taking care not to handle the hepatic artery. A loose temporary ligature was placed around the left branch of the portal vein from area a to area b. It was not always possible to perform this in a single manoeuvre without damage to the hepatic artery and local structures, so the ligature was passed from area b to area c and then passed under the portal vein to area a.

At this point, the hand picked islets were resuspended in 0.1 ml of HBSS containing 10% rat serum and aspirated through a 21G needle into a sterile 1ml syringe. The test tube was washed twice with 0.1ml of HBSS containing 10% rat serum, in order to transfer all islets from the test tube to the syringe. The needle on the syringe was changed to a 25G needle and the tip bent through 45°, in order to be more ergonomic for transplantation. The syringe was inverted and gently agitated, in order to collect the purified islets as close to the needle as possible. The temporary ligature around the left branch of the portal vein was tightened, diverting all blood flow into the right branch, and the 25G needle was inserted into the portal vein well below the bifurcation. The islets were slowly injected into the portal vein and blood was aspirated and re-injected three times to wash the syringe of any remaining islets. The temporary ligature was removed, restoring portal blood flow to both branches of the portal vein, and the needle removed. The portal vein was tamponaded with a cotton wool bud for at least 3 minutes or until haemostasis was achieved. The bowel was returned to the abdomen and the wound closed in layers with 4/0 vicryl. Five millilitres of normal saline was left in the peritoneal cavity for fluid replacement and sub-cutaneous Temgesic was administered as pain relief. The rats were transferred to a clean cage and placed in a warmed recovery suite for 48 hours postoperatively. Rats were allowed free access to standard rat chow and water. Blood glucose levels and weights were recorded at specified time points after transplantation.

Figure 2.1:
Method for right
branch
transplantation of
islets.



Main branch portal vein transplants (used for initial validation of the transplant technique) were performed in a similar manner to above description but xiphisternum removal, micro-dissection and use of a ligature around the portal vein was not performed.

2.2.6 Intra-peritoneal glucose tolerance test

Rats were anaesthetised with 24mg (0.4ml) of pentobarbitone (Rhone Merieux) ip. After deep anaesthesia was achieved, the tip of the rat's tail was amputated and blood expressed. One drop of blood was used for measurement of blood glucose, as above, and a further 6-7 drops were collected in a 500µl eppendorf, spun at 15,000 x g in a microcentrifuge RT and the plasma stored at -20°C. The rat received 2g/Kg of dextrose intra-peritoneally and further samples were taken at 15, 30, 60, 90 and 120 minutes. After 120minutes, the animals were humanely killed by rising inspired carbon dioxide concentration. Death was confirmed by cervical dislocation.

2.2.7 Retrieval of liver and pancreas

After death was confirmed, as above, the liver and pancreas were retrieved from the cadavers. A midline skin incision was made from xiphisternum to pelvic region and the abdominal cavity was opened. The liver was gently retracted inferiorly with two fingers and the liver was carefully dissected out of the rat.

2.2.8 Fixation and embedding of livers

The livers of transplanted rats were examined histologically for morphology or the expression of angiogenic factors by in situ hybridisation. Both techniques require different methods for the processing of the tissue.

2.2.8a Paraffin-embedded sections

Livers were retrieved as above and were divided into lobes supplied by the right branch of the portal vein (RB) and those supplied by the left branch (LB). The LB tissue was then divided into equal sections of 4mm thickness and placed into 6 separate cassettes. The RB tissue was also divided into sections of roughly 4mm thickness and placed into 3 separate cassettes. The right anterior lobe was placed in a single cassette and the right posterior lobe was divided antero-posteriorly before placing each section in a separate cassette. The tissue sections were kept in 4% neutral buffered paraformaldehyde for a further 2 – 4hours until processed in an ASP 300 automated tissue processor (Leica). After processing, the tissue was embedded in paraffin and kept until sectioned. The tissue used for haematoxylin and eosin staining were initially incubated for an additional 24 hours in 4% neutral buffered paraformaldehyde prior to processing.

2.2.8b Frozen livers for in situ

Livers were retrieved as above and fixed in 4% paraformaldehyde overnight. The livers were then freeze protected by incubating in sterile autoclaved DEPC treated 20% sucrose solution for six hours. The tissue was then transferred carefully to aluminium foil boats, covered in Tissue TEK, OCT compound (Sakura Finetek, Europe), frozen on carbon dioxide ice and transferred to a -20°C freezer until needed.

2.3 Histological analysis of transplanted islets

2.3.1 Haematoxylin and eosin staining

Paraffin-embedded livers were sectioned at 4µm thickness, placed onto water at 40°C and floated onto plain glass slides. The slides were baked on a 65°C hot plate for 10 minutes and processed through an automated slide staining system which incorporated dewaxing, rehydration, haematoxylin and eosin staining and dehydration. The slides were automatically cover slipped with a xylene-based compound and visualised immediately.

2.3.2 Immunohistochemistry techniques

Paraffin-embedded livers were sectioned at 4µm thickness, placed onto water at 40°C and floated onto TissueBond (Abcam Ltd, Cambridge, UK) coated slides. The slides were transferred to a humidified incubation box and kept overnight at 37°C. The tissue was dewaxed in 2 washes of xylene, passed through graded alcohol concentrations (100%, 95% and 70%) to water and washed in running water. Antigen retrieval was carried out by heating the slides in a microwave oven whilst immersed in buffer (table 2.1). The slides were cooled in running water, to avoid snap drying, and washed in tris-buffered saline (TBS). The slides were marked with a hydrophobic edging pen (Vector Laboratories, Peterborough, UK) and incubated with primary antibody diluted in 0.05M TBS, as per table 2.1. All antibodies were sourced from Vector Laboratories. After 1 hour, the slides were washed 3 times in TBS and incubated with a biotinylated secondary antibody for 30 minutes. The slides were washed a further 3 times and incubated with streptavidin-peroxidase complex (Vector Laboratories) for 30 minutes before washing again in TBS and then incubating with DAB (Vector Laboratories) for between 5-10 minutes while watching for brown colour development. After a further wash with TBS, the slides were counter stained with Mayer's Haematoxylin, incubated in acid-alcohol solution to wash out cytoplasmic haematoxylin and placed into blueing solution. After washing in water, the slides were dehydrated through graded alcohols, cover slipped with a xylene-based compound and imaged under a light microscope.

Table 2.1: Antigen retrieval and dilution of antibodies for immunohistochemistry.

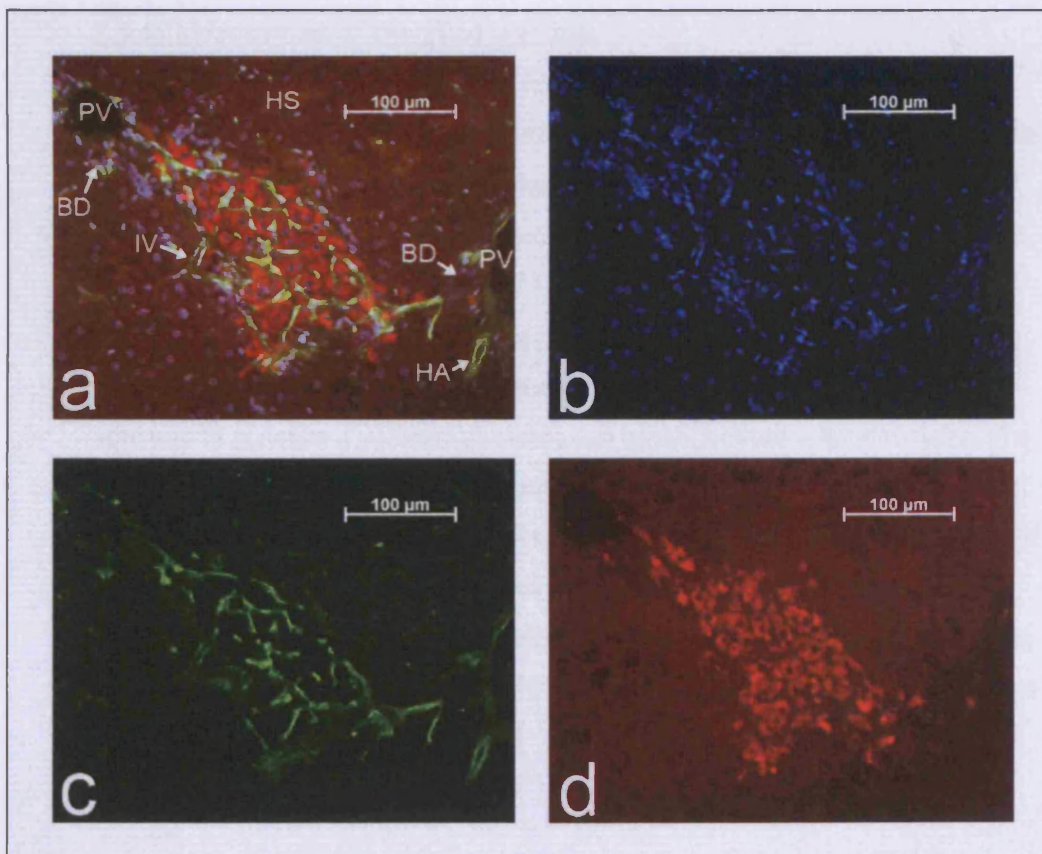
Antibody directed against	Dilution	Antigen retrieval technique	
		<u>Microwave time</u>	<u>Buffer</u>
CD31	1/100	20 minutes	Tris EDTA
Factor VIII related antigen	1/1000	10 minutes	Citrate
CD34	1/50	10 minutes	Citrate
Insulin	1/100	10 minutes	Citrate

2.3.3 Combined endothelial and insulin staining

Paraffin-embedded livers were sectioned at 4µm thickness, placed onto water at 40°C and floated onto Vectabond (Vector laboratories, UK) coated slides. The tissue was air dried at room temperature overnight, dewaxed in 2 washes of xylene, passed through graded alcohol concentrations (100%, 95% and 70%) to water and washed in TBS. The slides were marked with a hydrophobic edging pen (Vector Laboratories) and incubated overnight at 37°C in 0.1U/ml neuraminidase V (Sigma Aldrich, UK) diluted in acetate buffer (pH 5.0) supplemented with calcium chloride (see Appendix 1). Slides were washed in TBS x 3 and blocked by incubating for 1 hour with TBS containing 1:100 sheep serum (Sigma Aldrich). Primary incubation was overnight at 4°C in TBS supplemented with 0.1% (w/v) BSA containing 20µg/ml of biotin conjugated BS-1 (Sigma Aldrich) and 1:500 mouse anti-insulin antibody (Sigma Aldrich, UK). The slides were washed 3 times in TBS and then incubated with phosphate-buffered saline supplemented with 0.1% sodium azide containing 20µg/ml streptavidin-FITC (Sigma Aldrich) and 1:500 sheep anti-mouse antibody conjugated with rhodamine (Immune Systems Ltd, Paignton, UK). Slides were washed a further 3 times in TBS, counterstained for 3 minutes with methanol containing 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) 5µg/ml and washed a final 3 times in TBS before cover-slipping with Citifluor anti-fade agent (Agar Scientific, UK).

The slides were imaged with a Zeiss Axioskop 2 fluorescent microscope, photographed with a Zeiss MRm monochrome camera and processed with Zeiss Axiovision software. The final tricolour image was composed by overlaying the three colour images; green- for endothelium, red for insulin and blue for nuclei (see figure 2.2)

Figure 2.2: Formation of the tricolour image for the dual BS-1 and insulin technique. The final image (a) was created using Zeiss Axiovision software by overlaying the three consecutive images of blue- DAPI stained nuclei (b), green- FITC stained endothelium (c) and red- rhodamine stained insulin (d). This figure shows a single islet which spans between two portal triads. The labelled structures are: PV- portal venule, HA- Hepatic arteriole, BD- bile ductule, HS- hepatic stroma, IV- islet vasculature. All further images in the thesis will be merged tricolour images of islets.



2.3.4 Quantitative histological analysis

Quantitative analysis of JPG images obtained from the imaging of transplanted islets was performed using Image J software (freely available from the National Institutes of Health website, USA). Only islets with an area over $1000\mu\text{m}^2$ were used for analysis, in order to reduce the over/underestimation of endothelium and vascular density.

2.3.4a Scaling of images

Images were taken of a haemocytometer grid at the defined magnifications used for imaging islets (100x, 200x and 400x). The images were processed with Zeiss Axiovision software to obtain a scale for the number of pixels per μm , for each magnification. Four repeat measurements were used to reduce error in scale measurement. Zeiss Axiovision software was used to apply scale bars to images.

2.3.4b Measurement of engrafted islet area

JPG images of engrafted islets were imported into the Image J software. The scale was set within Image J (as derived from above calculations) and the outline of the insulin area of the islet (red) was drawn around, as shown in figure 2.3A. The area was calculated by the Image J software and displayed in μm^2 .

2.3.4c Measurement of percentage vascular area of islets

The images from islet area analysis were further manipulated within Image J software to generate a percentage estimation of vasculature within the islet. The image with outlined islet area was converted into a JPG stack and selected for the green channel; which will highlight only the signal for endothelium. A threshold for the green image was set (as shown in figure 2.3B) and the area of endothelium within the measured area was calculated in μm^2 . The vascular area was then divided by the total area of the islet to produce a percentage of the islet which was vascular endothelium.

2.3.4d Measurement of vascular density

The vascular density of islets was measured by two separate methods. The number of vessel branches was measured manually using the cell counter plug-in within Image J, as shown in figure 2.3C. The generated value for the number of vessel branches was then divided by the total islet area, as estimated above. It is possible that this methodology could be open to bias because a single vessel branch was only counted once, no matter what the length. Therefore a further method was used which would take into account the contribution of length of vessels. A grid of $225\mu\text{m}^2$ was applied to the islet image and vessels were counted within the islet if they crossed the upper or left hand edge of a grid, as shown in figure 2.3D. The generated value was then divided by the islet area to generate a value for vascular density.

2.3.4e Calculation of the branching index

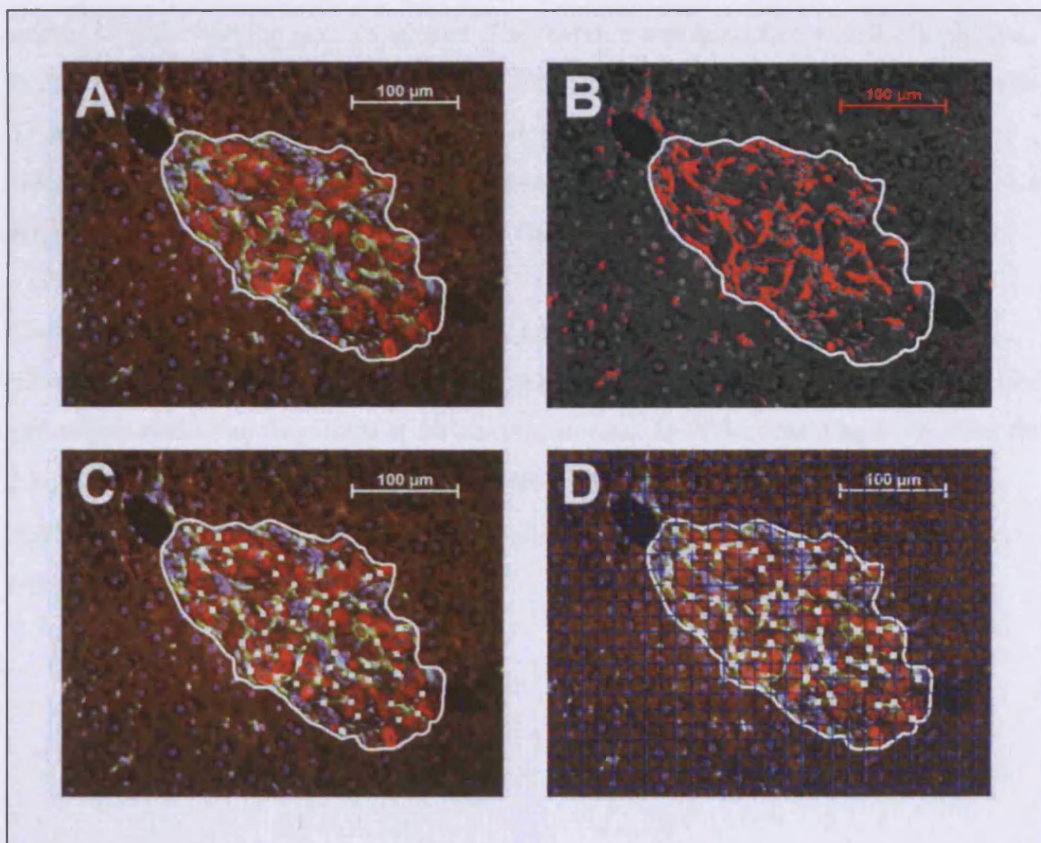
Two different techniques are used to estimate the vascular density of islets. The first method measures the number of vascular branches for a given sized islet and the second uses a grid technique to measure vessels within the islet. These two different techniques will give different information of the vascular architecture within an islet. If an islet has a small number of vessels with no branches, the first method will score the density low but, when using the grid method, the vessels will cross the grid at a number of points and the score will be relatively higher. Therefore, islets with vessels that have few branches will have a relatively lower score on the vessel counting method but higher score on the grid method. Islets with vessels that have a much higher number of branches (i.e. a glomerular type network of vessels) will have more comparable estimations for both techniques. This observation was further extrapolated to form a “branching index”, which would give an estimation of the number of vessel branches within the imaged islet. The branching index was calculated by dividing the vascular density using the vessel branch counting method by the vascular density calculated using the grid method. If the number of vessel branches within islets

increases, the branching index will have a higher score and quantify whether the islets formed a new vascular network.

2.3.4f Measurement of vessel size

High magnification images (400x) only were used for the estimation of vessel diameter. Zeiss Axiovision software was used to analyse the image by measuring the diameter of vessel lumens on a pre-scaled image. The measurements were made perpendicular to the vessel walls and only vessels within the image with clear delineation of the lumen (parallel “tramline” staining of both vascular walls of the vessel) were measured.

Figure 2.3: Methodology for assessing islet area (A), percentage vascular area of an islet (B) and vascular density (C and D).



2.3.5 Fluorescent in-situ hybridisation of the Y chromosome

Digoxegenin was directly incorporated into the Y chromosome probe from the plasmid using a PCR Dig incorporation kit (Roche Applied science, Lewes, UK) and internal primers. The PCR product was purified using Quiaquick PCR purification columns (Quiagen, Crawly, UK) and aliquots were checked for size by gel electrophoresis and quantity using ultraviolet absorbance spectrophotometry in a Gene-Quant (Pharmacia Biotech, Cambridge, UK) analyser (see below). The digoxigenin incorporated probes were resuspended in HybMix (Sigma Aldrich, UK) at 40-60ng/ μ l and heated to 100°C for 2 minutes, in order to denature the probe. The probes were cooled on ice and used immediately.

Paraffin embedded liver tissue was sectioned at 7 μ m, dewaxed with xylene and passed through graded alcohol to water before washing in PBS. The slides were marked with hydrophobic pen and incubated with 0.4% pepsin (Sigma Aldrich) in 0.1M hydrochloric acid at 37°C for varying periods of time. The reaction was quenched with 0.2% glycine in 2xPBS for 2 minutes before washing in PBS and post fixing in 4% paraformaldehyde. Twenty microlitres of the HybMix/probe solution was applied to the tissue. The slides were coverslipped and sealed with heat resistant glue (Evostick, UK) and incubated on a hot plate for 10 minutes before transferring to a moist incubator at 50°C overnight.

The next day, the coverslips were removed and tissue washed in 1xSSC at 37°C for 5 minutes. The slides were washed in PBS and subsequently incubated with FITC labelled anti-digoxegenin Fab fragments at 1:100 concentration in PBS containing 0.5% BSA for 2 hours. The slides were washed again in PBS, counterstained with 5 μ g/ml DAPI in methanol and coverslipped with Citifluor antifade reagent (Agar Scientific). The slides were imaged as above.

2.4 In-vitro desferrioxamine treatment studies

Isolated islets were treated with the iron chelating agent desferrioxamine (DFO) and investigated for the expression of key angiogenic factors, viability, apoptosis, glucose stimulated insulin release and insulin content.

2.4.1 Effect of desferrioxamine treatment on expression of VEGF and bFGF protein in cultured islets

Islets were isolated and washed in CMRL culture media containing foetal calf serum, penicillin/streptomycin, HEPES buffer and L-glutamine (CMRL) (see Appendix 1). Aliquots of islets were hand counted under a stereoscopic microscope and transferred to 6 well, flat-bottom culture plates. Each aliquot was covered with either 2ml of CMRL alone (control cultured) or 2ml of CMRL containing increasing concentrations of DFO. The concentrations of DFO used were 10 μ M (DFO 10), 100 μ M (DFO 100) or 1000 μ M (DFO 1000). The islets were cultured overnight under standard conditions in a humidified incubator at 37°C with 5% carbon dioxide. Hypoxic control islets were cultured in open plates containing CMRL within a modular incubator chamber (Billups Rothenberg, Del Mar, California, USA) which had been gassed for 20 minutes with a 1% oxygen, 5% carbon dioxide and balance nitrogen gas (BOC Ltd, Surrey, UK), an ambient oxygen concentration which had been shown to up regulate HIF-1 alpha in cultured islets (Moritz, Meier et al. 2002). Hyperoxic control islets were cultured as for the hypoxic controls but the modular incubator was gassed with 95% oxygen and 5% carbon dioxide (BOC Ltd, Surrey, UK).

After 16 hours, the islet cultures were inspected for evidence of contamination and integrity of cultured islets. If there was no evidence of contamination and islets were intact, the culture medium was completely aspirated, transferred to a 5ml polypropylene test tube and centrifuged at 800 x g for 5 minutes RT, to sediment any aspirated cellular material. The supernatant was carefully harvested and frozen at -20°C until analysed.

The remaining islets on the culture plates were covered with 2ml of the iron-containing medium M199 supplemented with foetal calf serum, penicillin/streptomycin, HEPES

buffer and L-glutamine (M199) (see Appendix 1). After a further 24 hours, the cultures were inspected and the culture supernatants were harvested and frozen as above. This process was repeated for a further 4 days in each culture.

2.4.2 Measurement of angiogenic growth factors by ELISA

The quantification of different growth factors was analysed by techniques according to the factor assayed. All ELISA plates were read on a 3550 microplate reader (Bio Rad) and analysed with Microplate Manager v2.0.2 software (Bio Rad).

2.4.2a Measurement of VEGF

Fifty hand picked islets were cultured as outlined above. The stored supernatants were defrosted and warmed to room temperature. The samples were vortexed to mix and analysed by mouse-specific VEGF ELISA (R&D Systems).

2.4.2b Measurement of bFGF

Three hundred hand picked islets were cultured overnight. The supernatants from the cultures were harvested the next day and islets were then washed twice in PBS before resuspending in 250µl of lysis buffer #11. The lysing cells were incubated on ice for 40 minutes before repeated aspiration through a 25G needle to shear DNA. Cell lysates were analysed using a human specific bFGF ELISA (R&D Systems).

The bFGF expression from lysed islets was corrected for total protein concentration within lysates, in order to correct for cell loss during the washing steps prior to lysis, and expressed as pg of bFGF per g of total protein. Total protein estimation was performed using a Lowry technique, as outlined below.

2.4.2c Measurement of HIF-1α

Culture conditions and sample preparation were similar to that for bFGF. The samples were analysed using a human/mouse HIF-1α experimental ELISA kit (R&D Systems).

2.4.2d Measurement of total protein concentration from cell lysates

Twenty microlitres of cell lysate, from above, was diluted in 980µl of double distilled water. Standard samples of known protein concentration were prepared from bovine serum albumin (Roche Applied Science, UK) and made up to 1ml. The standard concentrations of protein were 0, 5, 10, 20, 40, 60, 80 and 100µg/ml. The standards and samples were mixed with Lowry solution C, a solution made from a 100:1 mixture of Lowry solution A and Lowry solution B (see appendix 1). The samples and standards were incubated for 10 minutes at room temperature before adding 0.15ml of 2N Folin-Ciocalteu solution and incubating at room temperature for a further 45 minutes.

The standards and samples were read at 660nm on a Lambda 3B spectrophotometer (Perkin Elmer, UK). The standards were used to calibrate the spectrophotometer and sample concentrations were calculated by comparison to the standard curve.

2.4.3 Effect of desferrioxamine treatment on angiogenic factor gene expression

Islets were isolated from adult male Sprague Dawley rats, as above. The islets from a single donor were washed in CMRL and cultured overnight either in CMRL, DFO100, DFO1000 or CMRL under hypoxic conditions (1% oxygen), as above. The next day, islets (400-500 per experiment) were hand picked under a stereoscopic microscope and used for the extraction of RNA, as detailed in the molecular section below. Hand picked fresh islets were used as controls.

2.4.4 Effect of desferrioxamine treatment on islet viability

The viability of fresh, control overnight cultured (CMRL), desferrioxamine treated (DFO 10, DFO 100 and DFO 1000) and hypoxic islets was assessed by direct visualisation of whole islets and by flow cytometry on the cells from dispersed islets. Fresh hand picked islets treated with 150µM hydrogen peroxide were used as positive controls for cell death.

2.4.4a Flow cytometry method on dispersed islets

Fifty hand-picked islets were dispersed by incubation with acutase (Innovative Cell Technologies, California, USA) in a polypropylene test tube. The islets were initially incubated static with 1ml of Acutase at 37°C for 10 minutes, followed by a further 5 minutes of incubation at 37°C with gentle pipetting of the cell suspension using a 1ml pipette. The dispersion was visualised under a stereo microscope and the reaction was stopped when the islets were fully dispersed by adding 1ml of cold phosphate buffered saline (PBS) containing 10% new born calf serum. The resultant cell suspension was pelleted by centrifugation at 800 x g RT for 5 minutes, the supernatant removed and the cells gently resuspended by tapping the end of the tube. The resuspended cells were stained by adding 2.5µl of 250µg/ml solution of propidium iodide and incubating in the dark at room temperature for 10 minutes. The mixture was diluted with 500µl of PBS and analysed on an Epics XL flow cytometer (Beckman Coulter). Non-viable cells were stained by propidium iodide and the signal picked up on the FL3 sensor. The percentage viability of dispersed cells was calculated as the percentage of non-stained cells counted compared with all cells counted. The positive control for cell death was islets incubated in 150µM hydrogen peroxide prior to dispersal. Each sample was analysed in triplicate.

2.4.4b Direct visualisation of whole islets

Ten intact islets were hand picked in 100µl of culture medium and placed onto untreated glass slides (Sigma Aldrich). The cell suspension was stained with 1µl of acridine orange (0.02mg/ml) and counterstained with 2.5µl of propidium iodide (250µg/ml). The solution was gently mixed with the tip of a pipette, incubated for 5 minutes in the dark, cover slipped and visualised immediately on an Axiophot fluorescence microscope (Carl Zeiss, Germany). Images were acquired with stimulation by mercury light and filters for green and red light fluorescence, using a TS100 digital camera (Olympus) attached to the eyepiece of the microscope and stored for analysis. The pictures were analysed with Image

J cell counting software (NCBI) to assess the number of viable cells (positive green fluorescence) compared with non-viable cells (propidium iodide/red positive fluorescent nuclei). The assessment was repeated at least four times to gain a representative sample of the population tested.

2.4.5 Effect of desferrioxamine treatment on apoptosis of islets .

Apoptosis was assessed by Annexin V (Roche Applied Science, UK) and 7AAD (Invitrogen, UK) staining of dispersed cells on a flow cytometer, modified from the technique suggested by Cattani et al. (Cattani, Berney et al. 2001). Hand-picked islets were dispersed by incubation with acutase (as described above). The cells were centrifuged for 5 minutes at 800 x g RT and washed 3 times in Dulbecco's A PBS (without calcium or magnesium to avoid cell clumping). The dispersed islets were stained with Annexin V from the apoptosis assessment kit (Roche Applied Science, UK) and 7AAD (Invitrogen, UK) and resuspended by vortexing. The cell suspension was diluted in the phosphate based buffer provided by Roche Applied sciences and assessed on an Epics XL flow cytometer (Beckman Coulter, UK).

Positive controls (150 μ M hydrogen peroxide treated islets) and fresh islets were used for the set up of the flow cytometer. Single stained cells, either annexin V or propidium iodide, of either fresh or hydrogen peroxide treated groups were used to set the voltages and compensation on the flow cytometer.

2.4.5a Isolation and staining of peripheral blood mononuclear cells

The flow cytometry method for apoptosis assessment of disrupted islets proved problematic. In order to assess the methodology and check the function of the flow cytometer in non disrupted cells, peripheral blood mononuclear cells were used as an easy to access cell line with known cell surface markers.

Venous blood was obtained by standard venepuncture techniques and collected into heparinised syringes. Blood was mixed in equal quantities with RPMI 1640 (Life Technologies Ltd, UK) and layered onto Lymphoprep (Axis-Shield, UK) in

30ml polystyrene tubes (Greiner Bio-one Ltd, UK). The blood/density gradient mixture was centrifuged for 20minutes at 2000 x g RT and the interface harvested and diluted further in RPMI. The resultant peripheral blood mononuclear cells were washed three times in PBS and stained with annexin/propidium iodide as above. Apoptosis was induced as above with a 1 hour incubation in 150 μ M hydrogen peroxide, prior to staining.

A reliable positive control was required for assessment of flow cytometer function. The T cell marker CD4 was used for this purpose and freshly isolated peripheral blood mononuclear cells were stained with CD4-FITC antibody (Sero-Tech, UK) in PBS and washed a further three times in PBS before resuspending and analysing.

2.4.6 Effect of desferrioxamine treatment on the glucose related function of islets

The glucose stimulated insulin release and islet insulin content was assessed to gain an indication of the effect of desferrioxamine treatment on the metabolic function of islets.

2.4.6a Glucose stimulation test

Aliquots of ten islets were hand picked under a stereo microscope and placed into 2ml eppendorfs. One millilitre of Krebs ringer solution supplemented with 20mM Hepes buffer (pH 7.4) and 2% bovine serum albumin containing either 1.67mmol/l glucose (basal) or 16.7mmol/l (stimulated) was added to the islets and incubated for 1 hour at 37°C. The eppendorfs were centrifuged at 1000 x g for 10 minutes, to sediment cellular debris, and 400µl of supernatant harvested and frozen at -20°C for analysis of insulin content at 1:10 dilution by a rat specific insulin ELISA (Mercodia AB, Uppsala, Sweden). The stimulation index was calculated by dividing the stimulated insulin concentration by the basal insulin concentration. The islet pellet was frozen in the remaining Krebs Ringer solution and stored at -20°C for further analysis of insulin content.

2.4.6b Assessment of insulin content of islets

The pelleted islets from static glucose stimulation tests were thawed and vortexed to resuspend islets. The islet suspension was sonicated twice for 10seconds at 18microns in a Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, UK). The resultant mixture was re-vortexed to mix and analysed for insulin content at 1:500 dilution by a rat specific insulin ELISA (Mercodia AB). The results were expressed as insulin content per islet.

2.5 RNA molecular techniques

The expression of angiogenic growth factors was assessed at both protein and message level. The assessment of mRNA required a number of techniques, which will be described here.

2.5.1 Isolation of RNA

Hand picked islets were washed in CMRL and centrifuged at 800x g for 5 minutes RT. The supernatant was removed and the islets recentrifuged with further total removal of all culture medium. The islets were lysed with 1ml of TRIzol reagent (Invitrogen, UK) by active pipetting with a 1ml pipette and then transferred to a clean, RNase free 1.5ml eppendorf. The homogenised samples were incubated static for 5 minutes at room temperature and then 200µl of chloroform were added. The resultant mixture was vigorously agitated for 2 minutes before centrifuging at 12,000 x g for 15 minutes at 4°C. After centrifugation, 400µl of the upper aqueous phase of the supernatant was carefully removed without disturbing the interface (which contains sheared genomic DNA) and transferred to a clean RNase free eppendorf. 500µl of isopropanol was added, to precipitate the RNA, and the solution was incubated for ten minutes at room temperature before centrifuging at 12,000 x g for 10 minutes at 4°C. The supernatant was carefully removed and the resultant RNA pellet washed in 1ml of 75% ethanol. The ethanol and RNA solution was recentrifuged at 7,500 x g for 5 minutes at 4°C, the supernatant completely removed, recentrifuged and any remaining supernatant completely removed before air drying the pellet. After drying, the RNA pellet was resuspended in 50µl of molecular biology grade water (Sigma Aldrich, UK) and the resultant solution was analysed spectrophotometrically for RNA concentration and purity.

2.5.2 Analysis of RNA concentration and purity

The concentration and purity of RNA was estimated by ultraviolet absorbance spectrophotometry, using a Gene-Quant (Pharmacia Biotech, Cambridge, UK) analyser. Absorption of light was quantified at 260nm and 280nm to derive concentration and ratio values.

2.5.3 DNase treatment of RNA samples

RNA samples were treated with DNase to reduce any contamination of genomic DNA in the RNA samples, which might interfere with the real time PCR estimation of gene quantification.

One microgram of total RNA, as estimated above, was diluted in 8µl of water for molecular biology (Sigma, UK) and added to 1µl of DNase (Invitrogen, UK) and 1µl of RT buffer (Invitrogen, UK). The solution was vortexed to mix and incubated at room temperature for 15 minutes. 1µl of EDTA was added to stop the reaction and the solution was heated to 65°C for 10 minutes to denature the enzyme and stop it inhibiting the formation of cDNA in the next step. The sample was cooled on ice for 2 minutes before use.

2.5.4 Formation of cDNA

Complimentary DNA (cDNA) was formed from isolated total RNA using Taq-Man reverse transcription reagents (Applied Biosystems, Warrington, UK). Four hundred nanogrammes of total RNA were added to the suggested mixture of reverse transcriptase, RNase inhibitor, dNTPs, random hexamers, buffer, magnesium and water to a volume of 20µl in a PCR tube; as directed in the manufacturers instructions. The tubes were centrifuged, in order to collect all contents in the bottom of the tube, and transferred to a Gene Amp 9700 thermal cycler (Applied Biosystems, Warrington, UK). The machine performed a single cycle of 25°C for 10 minutes, followed by 48°C for 30 minutes (for cDNA formation by reverse transcriptase), followed by 95°C for 5 minutes (for denaturing of reverse transcriptase enzymes), followed by a final step of 4 °C until the cDNA was removed from the machine.

2.5.5 Design of real time PCR primers

The messenger RNA (mRNA) sequences for the required angiogenic factors were retrieved from the National Centre for Biotechnology Information (NCBI) website. The sequences were blast searched back into the web site to check the identity of the sequences were correct and then compared to rat genomic sequences. The comparison with genomic DNA allowed the mapping of intron-exon boundaries within the mRNA

sequence. The mRNA sequence and intron-exon boundary information was compared with primer sequences used by other authors and used to create new primer pairs using Primer Express v2.0 software (Applied Biosystems, UK). Primers were selected from the list of primers suggested by the software by choosing pairs where one primer overlapped an exon-exon boundary and the other primer was based within the adjacent exon. Primer pairs were especially chosen when the overlapping primer had only 5 to 7 bases within the exon containing the non overlapping primer and when both primers had similar melting points. If a primer set fulfilling these criteria did not exist on initial searching of the sequence, the computer was directed to an area within the mRNA sequence which contained exon-exon boundaries that had been spanned with primers designed by other authors.

The VEGF primers were designed to span designated exon-exon boundaries within the VEGF sequence, in order to examine specific VEGF transcripts; as described previously in the rat (Tober, Cannon et al. 1998). The expression of all VEGF isotypes was examined by choosing a primer pair with one primer spanning the exon 2 to exon 3 boundaries. The VEGF 164 isotype in the rat is formed by alternate splicing of the gene and lack of expression of exon 6. Therefore a primer pair was designed with one primer spanning the exon 5 to exon 7 boundaries. The careful design of primer pairs allowed the examination of the expression of different VEGF splice variants.

When the primer set had been designed, the sequences were BLAST searched back into the NCBI website to confirm that the primers identified the correct gene and did not additionally identify a different gene. Once all these criteria had been met, the sequences were sent to Sigma Genosys (Sigma, UK) for creation of desalted primers. Primers were diluted to 100 μ M concentration with water for molecular biology, according to manufacturer suggestion, and then further diluted as corresponding forward and reverse primer pairs to a concentration of 2.5 μ M. This working concentration was used with small aliquots stored at -20°C until required.

All primers were validated and investigated before use, as outlined in chapter 3.

2.5.7 Real time PCR estimation of gene quantification.

The real time PCR method for estimating relative gene quantification was performed with a 7000 Sequence Detection System machine (Applied Biosystems, UK) and SYBR green (Applied Biosystems, UK) DNA binding dye. Beta actin was used as the control for relative quantification.

Two micro litres of cDNA was pipetted onto the side of a 0.2ml PCR tube and 1.5 μ l of 2.5 μ M forward and reverse primer (sequences as in table 2.2) and 9 μ l of water for molecular biology (Sigma, UK) were pipetted onto separate areas of the same tube. 12.5 μ l of SYBR green (Applied Biosystems, UK) was pipetted into the lid of the PCR tube and the cap firmly placed on the tube without disturbing the other solutions. The tubes were centrifuged immediately before placing into the 7000 machine, to avoid mixing until the very last opportunity (hot starting). The 2 duplicate cDNA amplifications were run in parallel with amplifications containing similar dilutions of total RNA and water controls (amplifications containing primers only- also known as no template controls). The RNA controls acted to highlight any amplification of genomic DNA with the primer sets and the water controls were present to highlight any DNA contamination of the solutions or plastics. The final dilution of total RNA in the control amplifications was similar to the final dilution of total RNA that would be expected to be found in the cDNA amplifications after reverse transcription. This matching of dilutions would allow direct comparison of the cDNA amplification plots to any amplification in control plots and therefore enable estimation of the severity or significance of any contamination. The final composition of the real time PCR amplification runs are outlined in table 2.3. In addition, certain primer sets (basic fibroblast growth factor, angiopoietin 1 and endostatin) required the addition of formamide 2%, to improve the specificity of binding of primers; as highlighted in table 2.2.

Table 2.2: Primer sequences for gene analysis by real time PCR and primers that required addition of formamide. (VEGF all, refers to the primers which analyse all isoforms of vascular endothelial growth factor.

Gene analysed	Forward primer	Reverse primer	Formamide 2%
Beta actin	TGAAAAGATGACCCAGATCATGT	AGGGACAACACAGCCTGGAT	
VEGF all	AGCCCATGAAGTGGTGAAGTTC	GGTCTCAATTGGACGGCAAT	
VEGF 164	GAGATGAGCTTCCTGCAGCATA	GCTCACAGTGATTTCTGGCTTT	
HGF	CAAAACAAGGTCTGGACTCACATG	CTCCCAGAAGATATGACGGTGTAA	
bFGF	GAAGAGAGAGGAGTTGTGTCCATCA	CTCTTCTGTAACACACTTAGAAGCCAGC	✓
ANG 1	AGAAGCAGAACTACAGGTTATATTAAAGG	TAGCATAAGGGCGCATTGTC	✓
Endostatin	CTGCAGGACCTCTACAGCATTG	CCTCATCCTTCAGGTTGACGAT	✓

Table 2.3: Composition of cDNA amplifications and control amplifications

Component	cDNA amplification	RNA amplification	Water control
cDNA	2µl	-	-
RNA	-	2µl	-
2.5µM primer pair	1.5µl	1.5µl	1.5µl
SYBR green	12.5µl	12.5µl	12.5µl
Water	9µl	9µl	9µl

The amplification mixtures were transferred to the 7000 Sequence Detection Unit and amplified by heating to 94°C for 5 minutes followed by 50 cycles of 94°C for 30 seconds, 60°C for 30 seconds (annealing), 72°C for 2 minutes (elongation). The run was finished with a dissociation point estimation for the individual PCR products. The cycling temperatures for primer sets that required formamide were changed to an annealing temperature of 64°C for 30 seconds followed by an elongation step of 72°C for 30 seconds, in order to improve specificity and reduce binding of genomic DNA.

The 7000 sequence detection system and ABI prism 7000 software calculated the normalised emission (R_n) of a sample by subtracting the emission signal of the SYBR green I from the emission signal passive reference dye ROX.

2.5.7 Validation of PCR primers.

After designing specific primers for real time PCR, the primers required thorough validation to confirm they were specific, only amplified a single product, did not dimerise and whether they would amplify genomic DNA.

2.5.7a Isolation of rat genomic DNA

Ten ml of blood was collected from 2 Sprague Dawley rats at the time of islet harvest and collected into citrated blood tubes (Sarstedt Monovette, Nuembrecht, Germany). The blood was transferred to a 50ml test tube and the cells were lysed and nuclear protein digested by using a blood and cell culture midi kit for genomic DNA isolation (Quiagen Ltd, UK). Briefly, 10 ml of blood was added to 30ml of ice cold water and 10ml of ice cold buffer containing 320mM saccharose, 5mM magnesium chloride, 10mM tris-hydrochloride and 1% triton X-100. The tubes were inverted to mix and incubated on ice for 10 minutes, to lyse all cells. The solution was centrifuged for 15 minutes 1300 x g at 4°C, the supernatant removed and the pellet rewashed in the saccharose buffer. The resultant pellet of nuclei was resuspended in 5ml of 800mM guanidium hydrochloride containing 30mM EDTA, 30mM tris-hydrochloride, 5% tween-20, 0.5% triton X-100, to lyse the nuclei, and digested with 95µl of 20mg/ml protease for 45 minutes, to strip DNA of any associated protein. The lysate was then applied to an equilibrated Genomic-tip (Quiagen) column (a resin based purification column) and washed with 1M sodium chloride /15% ethanol solution containing 50mM MOPS at pH 7.0. The column was eluted with 5 ml of 1.25M sodium chloride /15% ethanol solution containing 50mM tris-hydrochloride at pH 8.0. The eluted DNA was precipitated with 0.7 volumes of isopropanol at room temperature, to avoid precipitation of salts, and centrifuged at 10,000g for

20minutes at 4°C. The supernatant was aspirated, the pellet air dried and the DNA resuspended in 50µl of TE. DNA was quantified spectroscopically.

2.5.7b Validation of primers

The primer pairs were used for standard PCR amplification with cDNA, genomic DNA and water as template, to investigate their ability to amplify the different templates under specific conditions.

Two hundred nanogrammes of cDNA or genomic DNA was amplified by PCR with 10µl of Reddymix (AB Gene, Epsom, UK) pre-mixed PCR solution and 1.5µl of 2.5µM primers in a 9700 thermal cycler (Applied Biosystems, Warrington, UK), at a final volume of 20µl. The thermal programme was 94°C for 3 minutes, to denature the DNA, followed by 50 cycles of 94°C for 30 seconds, 55°C for 30 seconds (annealing), 72°C for 2 minutes (elongation). The run was finished with a further 5 minutes at 72°C before ending at 4°C. Annealing temperatures of 57°C, 60°C and 63.5°C were also explored.

Validation of primers was also carried out under real time PCR conditions, as outlined below.

2.5.7c Assessment of PCR products

PCR product (10µl) was loaded onto a 2% agarose gel in TBE containing 5µg/ml of ethidium bromide. Superladder 100bp ladder (AB Gene) was used to estimate the product size and the gel run at 150volts, variable current, for 50 minutes. The gel was visualised by an ultra violet imaging camera, Gel Doc 2000 (Bio Rad, Hertfordshire, UK) and images were analysed on Quantity One (Bio Rad) software.

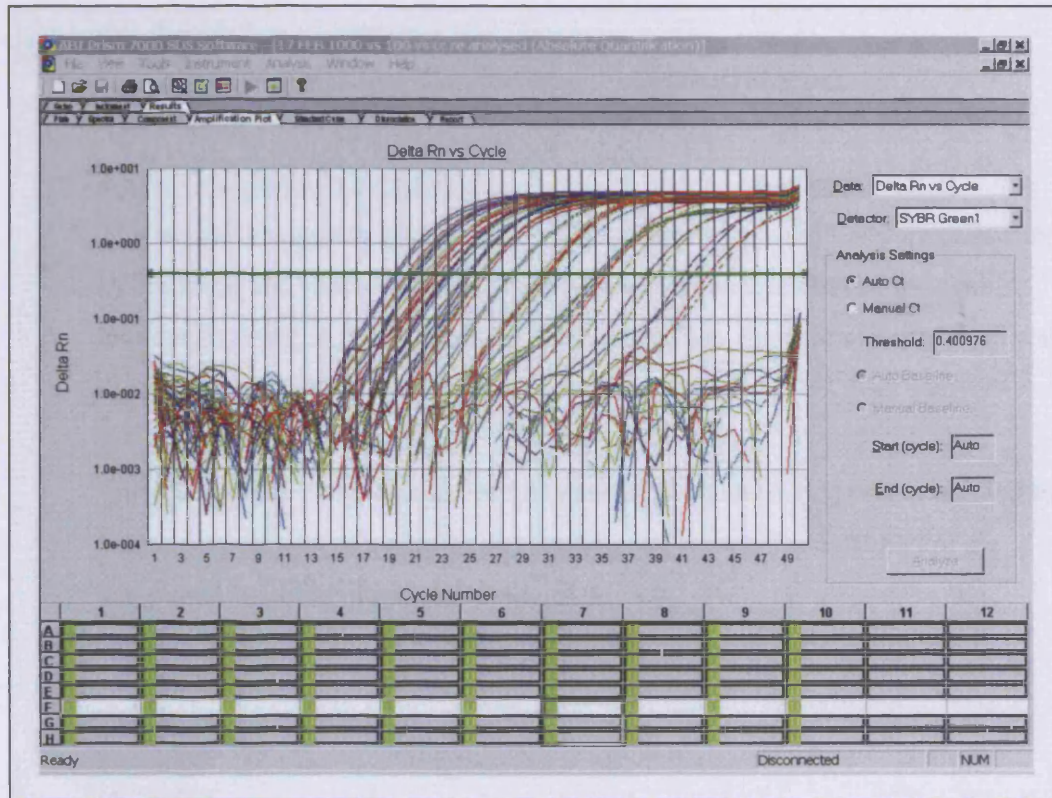
2.5.8 Analysis of amplification plots and relative fold gene expression

The raw data from the 7000 Sequence Detection Unit was collected at the time of amplification by ABI Prism 7000 (Applied Biosystems) software on a laptop associated with the sequence detection unit. The software displayed the amplification data graphically in the form of sigmoid plots of normalised emission signal from SYBR green DNA dye against cycle number (see figure 2.4). Analysis was only performed using plots which were sigmoid in form and parallel to the beta actin amplification plots used for reference. Sample amplification plots were excluded if the associated water or RNA controls showed evidence of amplification. In the rare case of amplification of RNA controls, seen only with occasional amplifications using endostatin, bFGF and ANG 1 primer pairs, the amplification plots were only used for analysis if there was over a 10 cycle difference in the Ct values between cDNA and RNA; corresponding to a 1000 fold difference in cDNA to genomic DNA amplification.

2.5.8a Calculation of threshold cycle (Ct) values

The threshold cycle (Ct) values were calculated using the ABI prism 7000 software. The selected amplification plots were analysed by setting the threshold at a point which bisected all the plots in their log phase of amplification (the green line in figure 2.4). This was often performed automatically by the software but occasionally had to be performed manually. The point of bisection of the amplification plot with the threshold line allowed the calculation of the cycle number at which the individual samples reached a similar stage of amplification, the threshold cycle (Ct). The calculated Ct values from all samples were collected for further comparative analysis.

Figure 2.4: Amplification data expressed as signal against cycle number when displayed using ABI Prism 7000 software. The green line indicates the point of analysis used for calculating Ct values for individual samples.



2.5.8b Calculation of intra sample differences in gene expression (ΔC_t)

The relative quantification of gene expression with real time PCR relies on having a reference to compare for fold analysis. The first step requires the analysis of the difference of gene expression within a sample, when compared to a reference. For this series of experiments, beta actin was chosen as the reference (housekeeping gene) because hypoxia has little effect on the expression of this gene, unlike GAPDH which is up-regulated by hypoxia (Escoubet, Planes et al. 1999; Zhong and Simons 1999) and hypoxia inducible factor (Graven, Bellur et al. 2003).

The difference in the expression of a gene within a sample (ΔCt) was calculated by subtracting the Ct value of the control gene from the Ct value of the target gene (beta actin in this case).

$$\Delta Ct = Ct (\text{target gene}) - Ct (\text{control gene})$$

2.5.8c Calculation in inter sample differences in gene expression ($\Delta\Delta Ct$)

The relative changes in gene expression under differing conditions are calculated by analysing the gene expression during the experiment relative to the gene expression found in control experiments. In this case, the control experiment was the gene expression of control islets.

The relative difference in inter sample gene expression ($\Delta\Delta Ct$) was calculated by subtracting the ΔCt of a gene in an experimental sample from the ΔCt of the same gene in a control sample.

$$\Delta\Delta Ct = \Delta Ct (\text{gene y/control sample}) - \Delta Ct (\text{gene y/experimental sample})$$

The ΔCt for a gene in the control sample (either fresh islets or control cultured) was determined by calculating the average ΔCt from all the PCR runs of that particular gene. This ΔCt was used as the control sample ΔCt for calculating the $\Delta\Delta Ct$ of that particular gene for each PCR run.

2.5.8d Calculating the relative fold change in gene expression

The $\Delta\Delta C_t$ value for a gene within an experiment is the difference in the number of PCR cycles between the expression of the gene in the experimental conditions relative to the control conditions. Therefore, given that each PCR cycle doubles the number of copies of the DNA specified by the primers, the fold expression difference between the two samples can be expressed as 2 to the power of the $\Delta\Delta C_t$.

$$\text{Relative fold change in gene expression} = 2^{\Delta\Delta C_t}$$

This assumption requires that the amplification kinetics of the experimental gene (gene y) and the control gene (beta actin) are comparable – the two sigmoid amplification plots are parallel. In addition, the threshold has to be set at a point where each cycle is producing a doubling of PCR product – most reliably during the middle exponential phase of the curves.

The final relative fold change in gene expression was calculated as an average of all analysis of a single gene under a specified condition (i.e. VEGF expression after DFO1000 treatment).

2.6 DNA molecular techniques

The initial project design required the use of in-situ hybridisation to examine the origin of endothelium with a Y chromosome probe and the site of growth factor expression using riboprobes. Although some of these techniques were not fully developed, the relevant sequences were sought, amplified and sequenced prior to use. This section will describe some of the common techniques used.

2.6.1 IMAGE clone selection

Sequences for the formation of riboprobes were chosen by searching the National Centre for Biotechnology Information (NCBI) website for appropriate and well matched Integrated Molecular Analysis of Genomes and their Expression (IMAGE) consortium clones. The clones were chosen to be either rat specific or mouse clones that were almost (greater than 90%) analogous to rat sequences.

2.6.2 IMAGE clones chosen

The following image clones were chosen for conversion into riboprobes:

Target molecule	IMAGE clone
VEGF	4934916
bFGF	2125620, 2689015
Ang 1	5353767
Ang 2	3660458
TGF β	5623078
Pro-Insulin	6430439,6432765

2.6.3 Rat Y chromosome probe

The rat Y chromosome probe was supplied as a gift from Dr B Hoebe (RIMV, Netherlands). The probe was obtained as a plasmid and transformed into E Coli - One shot™ (Invitrogen, Gronigen, Netherlands)

2.6.4 Broth amplification of plasmids

IMAGE clones were dilution streaked onto LB agar plates containing 50µg/ml ampicillin and incubated overnight at 37°C. Single colonies were picked from the plates with sterile microbiological loops and transferred to 2.5ml of LB broth containing 50µg/ml ampicillin and incubated overnight at 37°C with continual agitation.

2.6.5 Isolation of plasmid DNA

Two millilitres of resultant broth were harvested and centrifuged at 800 x g in 2ml eppendorfs for 5 minutes RT. The supernatant was removed, the pellet dispersed by vortexing and then resuspended in 200µl of GTE. The bacterial cells were lysed by adding 400µl of bacterial lysis buffer (see Appendix 1) and mixed by gentle inversion of the tube in order to avoid shearing chromosomal DNA. The cell lysate was incubated on ice for 10 minutes and neutralised with 200µl 3M potassium acetate. The tube was mixed by inversion and further incubated on ice for 10 minutes. The mixture was centrifuged for 10 minutes at 15,000 x g RT in a microcentrifuge, in order to precipitate chromosomal DNA and protein, and 700µl of supernatant containing plasmid DNA was decanted by pipette. The supernatant was transferred to a 1.5ml eppendorf and 1ml of cold absolute ethanol was added, to precipitate plasmid DNA. The mixture was incubated on ice for 30 minutes and centrifuged for 15 minutes at 15,000 x g RT in a microcentrifuge. The supernatant was carefully aspirated and the resultant pellet washed with 200µl of cold 70% ethanol and centrifuged again at 15,000 x g RT for 10 minutes. The supernatant was aspirated, the tube recentrifuged briefly and the remaining supernatant removed. The pellet was air-dried and then resuspended in 50µl of tris-EDTA (TE) containing RNase. This was kept at as a stock solution of DNA at -20°C and working concentrations of 1:200 dilutions in TE were taken from stock.

2.6.6 Glycerol stock of plasmids

The remaining 0.5ml of broth was mixed with 0.5ml 30% (v/v) glycerol in LB medium, to make a solution of 15% (v/v) glycerol in LB. The stocks were stored at -20°C.

2.6.7 Digestion of plasmids

Plasmids were digested to check that plasmid DNA was present. Plasmids were mixed as below in a 100µl PCR tube:

- 2µl of buffer (buffer B)
- 10µl of plasmid
- 1µl of HINDIII
- 7µl of distilled water

The tubes were incubated for 30-60minutes at 37°C. The digest was then mixed with 6µl of 5X loading dye and loaded onto a 1.5% agarose gel in TBE containing 5µg/ml of ethidium bromide.

2.6.8 Check of plasmids by PCR prior to broth amplification

Further single colonies from LB agar plates were transferred to 100µl PCR tubes containing 20 µl PCR buffer (Quiagen, UK) and heat treated for 10 minutes at 94°C to kill the bacteria and denature bacterial proteins. The resulting DNA was used as a template in PCR reactions to check the integrity of plasmids prior to broth amplification.

2.6.9 Polymerase chain reaction (PCR) amplification

Plasmid DNA was amplified by PCR with Reddymix (AB Gene, Epsom, UK) pre-mixed PCR solution and M13 (AB Gene) forward and reverse primers in a 9700 thermal cycler (Applied Biosystems, Warrington, UK). The programme was 94°C for 3 minutes to denature the DNA followed by 30 cycles of 94°C for 30 seconds, 57°C for 30 seconds (annealing), 72°C for 2 minutes (elongation). The run was finished with a further 5 minutes at 72°C before ending at 4°C.

2.6.10 Purification of PCR product

The PCR product was purified to remove excess primers and unincorporated bases using a Quiaquick gel centrifugation system (Quiagen GmbH, Germany).

2.6.11 Estimation of PCR product length

PCR product (10µl) was loaded onto a 1.5% agarose gel in TBE containing 5µg/ml of ethidium bromide. Superladder 100bp ladder (AB Gene) was used to estimate the product size and the gel run at 150volts, variable current, for 50 minutes. The gel was visualised by an ultra violet imaging camera, Gel Doc 2000 (Bio Rad, Hertfordshire, UK) and images analysed on Quantity One (Bio Rad) software.

2.6.12 Estimation of DNA concentration and purity

The concentration and purity of the PCR product was estimated by ultraviolet absorbance spectrophotometry, using the absorbance of 260nm light for estimation of DNA concentration and 280nm light as reference for protein contamination. Ratios above 1.8 were used as a cut off for uncontaminated samples. The samples were measured in a Gene-Quant (Pharmacia Biotech, Cambridge, UK) analyser.

2.6.13 Sequencing of PCR products

The PCR products were sequenced using the chain termination method, incorporating fluorochrome labelled di-deoxynucleotides and subsequent analysis by capillary gel electrophoresis in an automated genetic analyser. All products were sourced from Applied Biosystems, unless stated otherwise.

PCR product (20ng) was mixed with Big Dye Terminator v1.1 cycle sequencing solution and either M13 forward or M13 reverse primers. The reactions were cycled in a 9700 thermal cycler by heating to 96°C for 2 minutes followed by 30 cycles of 94°C for 10 seconds(denaturing), 50°C for 5 seconds (annealing) and 60 °C for 4 minutes(elongation). The final step brought the reaction temperature to 4°C until required.

The products were purified by ethanol precipitation and sequenced on an ABI Prism 310 genetic analyser (Perkin Elmer). The results were analysed on Gene Scan and Sequencing Analysis software to provide a sequence of the PCR products.

2.6.14 Confirmation of sequences of PCR products

The identity of the sequenced plasmid insert was confirmed by using the program BLAST (NCBI database) to compare with known sequences on the database.

2.7 In-vivo desferrioxamine treatment studies

2.7.1 Effect of transplanting desferrioxamine treated islets on reversal of diabetes

Islets were isolated as above, washed in CMRL, hand picked under a stereo microscope and transferred to a 90mm Petri dish for overnight incubation in 7ml of CMRL, DFO 100 or DFO 1000. The next day, the islet cultures were examined for contamination and integrity of islets. Two different doses of islets were transplanted, either a very marginal dose of 350 islets or a less marginal dose of 500 islets. The islets for transplantation were hand picked, washed in ice-cold HBSS and resuspended in 0.2ml of HBSS containing 10% Lewis rat serum. The islets were then transplanted into the right branch of the portal vein (as described in chapter 2). Body weight and blood glucose were measured daily for 2 weeks and weekly for a total of 11 weeks. After that time, the animals were humanely killed by a rising concentration of inspired carbon dioxide. The liver and pancreas were harvested and stored for subsequent histological analysis with dual insulin and endothelial staining.

2.7.2 Histological analysis of desferrioxamine treated and control islets

At the time of liver harvesting, the right lobes of the liver were divided into three sections and individually embedded in wax blocks. Each block was sectioned and two representative sections from different levels of each block were used for analysis. A total of six sections from each animal and eighteen sections from each experimental group were used for analysis. The sections were analysed for qualitative morphology and quantitative analysis of vascular density, graft area and vascular area.

2.8 Clinical islet transplantation

Clinical islet transplantation was undertaken in accordance with local ethics committee submission (appendix 3). The protocols used for recipient selection, islet isolation, transplantation and post transplant immunosuppression were in accordance with the original Edmonton protocol (Shapiro, Lakey et al. 2000). In brief, islet isolation was carried out on human donor pancreases with short cold ischaemic times using Liberase HI (Roche GmbH, Germany) or Collagenase NB/neutral protease mix (Serva Electrophoresis, Germany). Islets underwent a brief period of culture in modified CMRL culture media before being transplanted intra-portal via a transcutaneous and radiographically guided approach.

All patients received triple immunosuppression with Daclizumab (Roche Pharmaceuticals, Basel, Switzerland), sirolimus (Wyeth Pharma, USA) and low dose tacrolimus (Fujisawa, Japan), in accordance with the Edmonton protocol. Patients also received standard transplant prophylaxis, although our unit does not give cytomegalovirus (CMV) prophylaxis and patients were treated by preemptive screening by PCR followed by directed therapy. Patients were followed post transplant at regular intervals with regular screening for infection, drug toxicity/efficacy and diabetic control.

2.9 Statistical analysis

All data was collected and analysed using Microsoft Excel for Windows 2003 (Microsoft corp., USA). Simple inter-group comparative statistical analysis was performed with t-tests using the analysis tool pack within Microsoft Excel. More complex statistics and repeated measures were performed with repeated measures ANOVA and post hoc analysis using Statistica 6.0 for Windows (Stat-soft Inc., OK, USA). All data is expressed as mean plus one standard error, unless otherwise stated.

Chapter 3 – Results:

Validation and new techniques

3.1 Introduction

Pancreatic islets were originally discovered in 1869 by a Berlin medical student, Paul Langerhans (Langerhans 1937). Since the original description by Langerhans, pancreatic islets have been recognised to be a complex conglomeration of endocrine cells (Orci 1976) with an intricate vascular anatomy (Bonner-Weir and Orci 1982; Brunnicardi, Stagner et al. 1996). The complex structure and anatomy of islets enables the cells to communicate with each other and provide a coordinated response to glucose homeostasis (Weir and Bonner-Weir 1990). This structural conformation of islets appears to be important to their function and even after disruption, cultured islets cells tend to reaggregate into a similar pattern to that of native islets (Halban, Powers et al. 1987). Although disrupted islets can be transplanted and do reform a network of blood vessels, the revascularisation of disrupted islets is incomplete and clinical outcomes are worse when compared to non disrupted islets (Beger, Cirulli et al. 1998). Therefore, the use of β -cell lines or disrupted islets would not have provided sufficiently accurate data to study the revascularisation of transplanted islets and a source of fresh whole islets was required. The need for intact islets was further compounded by the requirement of a living recipient, in order to study the changes in endothelial architecture during the revascularisation of transplanted islets. Thus, an in-vivo model was required for experiments which examined islet revascularisation.

The isolation of islets from the whole pancreas is paramount to the success of post transplant outcomes (Nano, Clissi et al. 2005) and small changes to the isolation technique or consumables used can have a great impact on the function and survival of isolated rat islets (de Haan, Faas et al. 2004). The process of isolation and transplantation of pancreatic islets is complex and is not standardized between different investigator groups. Therefore, a certain amount of validation was required to optimise the isolation of pancreatic islets prior to transplantation. Once optimal islet isolation was

achieved, a suitable site for transplantation was required. Although a number of authors have used the renal subcapsular site for transplantation and easy retrieval of transplanted islets (Carlsson and Mattsson 1993; Carlsson, Palm et al. 2000; Vasir, Jonas et al. 2001), this model is not clinically relevant. The use of the more clinically relevant intra-portal site for transplantation has been limited by the poor retrieval of islets but the selective right branch transplantation model (Juszczak M 2003), developed within our unit, provided a method for circumventing this problem.

The original technique of right branch transplantation was designed for male to male transplantation of syngeneic rats. The model was further adapted in order to track the origin of engrafting islet endothelium and sex mismatched transplantation with Y chromosome in-situ hybridisation was undertaken for demarcation of endothelial origin. Although sex mismatching does not adversely effect the outcome of immunosuppressed human allogenic solid organ transplants (Ellison, Norman et al. 1994), there is evidence from human allogenic stem cell grafts that sex mismatching may induce antibodies to the minor histocompatibility antigens encoded by the Y chromosome (H-Y) (Spierings, Vermeulen et al. 2003; Miklos, Kim et al. 2004). Therefore, further validation was required to investigate whether the transplantation of syngeneic male rodent islets into non-immunosuppressed female rodent recipients would affect the outcomes after transplantation. In addition, the female recipients were smaller than male recipients and dose ranging experiments were required to evaluate the number of islets necessary to fully reverse streptozotocin induced diabetes in female recipients.

Islet transplant models provide useful clinical endpoints (changes in weight and blood glucose after transplantation) to assess the engraftment of islets but do not provide accurate assessment of the critical process of islet revascularisation. Histological assessment of islet grafts enables the visualisation of revascularisation but relies on accurate identification of structures to fully appreciate this important process. Direct visualisation of grafts under the kidney capsule (Carlsson, Palm et al. 2000) or dorsal skin fold (Vajkoczy, Menger et al. 1995) has allowed insight into revascularisation but are limited by graft clumping and lack of clinical relevance. Islets have been visualised

within the liver after transplantation but studies are limited by lack of structural definition (Griffith, Scharp et al. 1977) or the need for serial sectioning to define graft and vascular area (Carlsson, Palm et al. 2001). A new staining technique was required to simultaneously define graft area and vascular anatomy in order to accurately quantify graft revascularisation. A specific marker of islet vasculature (Mattsson, Carlsson et al. 2002) was used in combination with insulin staining in order to develop a novel dual staining method for accurately visualising intra-portally transplanted islets (Jones, Juszczak et al. 2005). The visualisation of transplanted islets with a dual staining technique had not been reported in the literature previously and optimisation of staining was required to gain accurate architectural definition of both endothelium and endocrine mass.

Although the clinical outcomes after transplantation are probably the most important, the in-vivo transplant techniques are influenced by multiple experimental factors and require large numbers to reduce the variability of the endpoint. In-vitro assessment of islets is important to reduce assessment variability by focusing on a single aspect and explore proof of concept. A number of the techniques used for the assessment of islets are based on standard culture and functional methods but further optimisation was required to customise islet viability and apoptosis assessments which were modified from reported techniques (Cattan, Berney et al. 2001).

The assessment of growth factors required optimisation and new primers had to be designed and evaluated before use in experimental studies. Although a number of molecular techniques were already established within the laboratory, the gene expression assessed in this project had not previously been evaluated within the group. For this reason, new primer sets were designed and had to be evaluated before use and a description of the process is provided in order to verify the accuracy of analysis.

This chapter describes the modification or adoption of non standard techniques and the processes required to establish novel methods.

3.2 Validation of common in-vivo techniques

The project required a number of common techniques which were based around the isolation and transplantation of islets. In order to perform these techniques it was necessary to standardise and validate the induction of diabetes with streptozotocin, blood glucose measurements, islet isolation and islet transplantation.

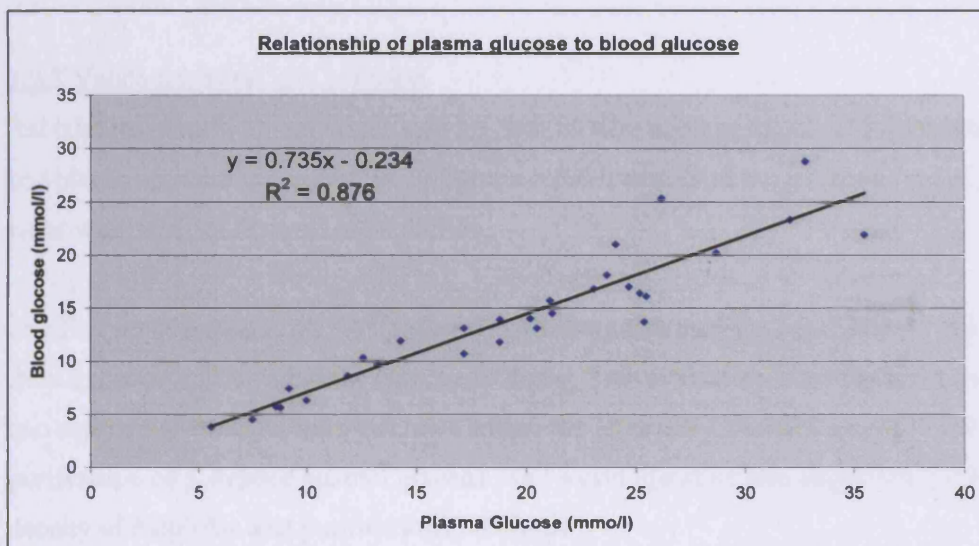
3.2.1 Validation of capillary blood glucose measurements

Diabetic and transplanted animals underwent regular estimations of blood glucose for verification of diabetes induction or monitoring of graft function. In order to reduce the stress and pain associated with repeated tail tipping and plasma glucose estimations, capillary blood glucose was measured using an Accu-Check Advantage II glucometer (Roche Diagnostics, Germany). As plasma glucose estimation is regarded as the gold standard for glucose measurement and the Accu-check was originally designed for human use, the method required validation.

Validation was achieved by collecting rat blood samples (in heparinised capillaries) at the same time as blood glucose measurement with the Accu-Check glucometer. Samples were collected during intra-peritoneal glucose tolerance tests and from a single non diabetic rat, to provide a range of glucose concentrations. Two male recipients and two female recipients were used as subjects. The rat blood samples were transferred to a 200µl eppendorf, centrifuged 10min x100g RT and analysed immediately on a Cobas Mira auto-analyser (Roche).

The capillary blood glucose measurements were lower than plasma levels, as expected, and within the range of 4.6 – 28.8mmol/l. Blood glucose measurements correlated well with the measured plasma estimations ($R^2 = 0.88$, figure 3.1) and therefore, this technique was adopted to estimate blood glucose concentrations

Figure 3.1: Correlation between blood glucose concentrations measured by the Accu-Check Advantage II glucometer and plasma glucose concentrations measured by the Cobas Mira auto-analyser.



3.2.2 Validation of diabetes induction.

Diabetes was induced in 13 animals (3 male Lewis rats and 10 female Lewis rats) with 55mg/Kg of STZ. The 3 male subjects were rendered diabetic with a reduction in mean body weight from 251.3g (± 10.9 g) to 224.3g (± 15.8). The average random blood sugar post STZ was 27.4mmol/l (± 3.2). Of the ten female subjects, 4 died within 3 days of diabetes induction, with signs of rapid weight loss, dehydration and coma. The remaining 6 female subjects became diabetic, with a reduction in mean body weight from 194.0g (± 2.21) to 174.8g (± 2.0) and an elevation in mean blood glucose to 30.3mmol/l (± 1.5).

All animals were rendered diabetic by 3 days post STZ injection and all, bar the 4 female animals who died, remained stable post diabetes induction for up to 10 days. The reason for the deaths in the female group was thought to be induction of severe diabetes due to a higher fat to total body mass ratio in female animals. Therefore, as STZ is lipophilic with a prolonged half life of active metabolites (130 hours in humans), the female rats receive a greater area under the curve exposure to STZ metabolites than

males and are likely to be rendered more severely diabetic. The dose of STZ was subsequently reduced to 50mg/Kg for female rats and diabetes induction in females was more predictable, without further loss of subjects.

3.2.3 Validation of rat islet isolation

Rat islet isolation is an important step for both in-vitro and transplant model experiments. In order to optimise the technique and prove reproducibility of the isolation process, islets were isolated from rat male donors.

The first set of experiments was undertaken to maximise the Ficoll purification of islets from exocrine tissue within the pancreatic digest. This evaluation was required because two separate techniques were available within the laboratory, purification on Ficoll and purification on a stepped dextran gradient, and world literature also suggested a lower density of Ficoll for islet purification (1.077g/ml).

Pancreatic digests from 2 Sprague Dawley rats were split into thirds and applied to three different ficoll densities of 1.082g/ml, 1.084g/ml and 1.086g/ml. The resultant purified preparations were examined under a stereo microscope for purity and yield of islets.

The purified islet fraction from the 1.082g/ml gradient was of high purity but low quantity while the fraction from the 1.086g/ml gradient had a high quantity of islets but was of low purity, which limited hand picking of islets. It was therefore decided to use a 1.084g/ml gradient and sacrifice some purity for a better yield of islets but not contaminate the preparation to the point at which hand picking of islets was hindered.

The next set of experiments examined the volume of collagenase used for distension and enzymatic digestion of the pancreas during isolation. The use of larger volumes of buffer provides better distension of the pancreas and may help separate the endocrine/exocrine interface but the higher pressure within the pancreas when delivering larger volumes of collagenase may lead to damage of the islet and infiltration of collagenase into the islet, leading to fragmentation and damage to the islet during digestion. Therefore,

experiments were undertaken using a standard dose of 5mg of collagenase P (Roche) dissolved in increasing volumes (3.3, 4 and 5ml) of HBSS. Islets were subsequently isolated and counted (Table 3.1).

Table 3.1: Comparison of islet yield when using different volumes of buffer infused with collagenase.

Volume of buffer used (ml)	Average number of islets isolated (+/- SE)	Number of isolations
3.3	499.9 (85.2)	6
4.0	366.8 (58.6)	4
5.0	270.5 (85.1)	6

These experiments showed that increasing the volume of buffer infused was associated with a stepwise reduction in yield, which was significant when comparing 3.3ml to 5ml volumes. Therefore, all further experiments were carried out using the 3.3ml volume of buffer for distension of the pancreas.

3.2.4 Validation of non selective intra-portal transplantation

The investigation of islet transplantation requires a robust model with reproducible endpoints. The next set of experiments examined whether the transplant technique met this criterion.

The remaining 9 diabetic animals (3 male Lewis rats and 6 female Lewis rats) all underwent main branch intra-portal islet transplantation. Out of these 9 animals, one female recipient died during surgery and the remaining animals had successful islet transplants. The three male recipients each received a mean of 644 (\pm 14.7) islets, (Table 3.2).

All animals had a fall in blood glucose post transplantation, although one recipient reverted to almost pre transplant blood glucose levels after 2 weeks. Two weeks after transplantation, the mean body weight of the three male recipients had risen from 224.3g

(± 15.7) pre-transplant to 245.7g (± 12.5) and the mean random blood glucose had fallen from 27.4mmol/l (± 3.2) pre transplant to 16.0mmol/l (± 4.3) (Table 3.2).

The 5 female recipients received a mean of 691.2 (± 135.9) islets transplanted via the main branch of the portal vein (Table 3.2). All 5 recipients had a reduction in blood glucose. After 2 weeks post transplantation, the mean body weight increased from 173.8g (± 3.0) pre transplant to 180.3g (± 6.0) and the mean blood glucose had fallen from 32.0mmol/l (± 1.0) to 9.7mmol/l (± 2.0).

Table 3.2: Weight and blood sugar of rats prior to and after transplantation during experiments to validate sex matched and sex mismatched transplant model.

Sex of recipient	Wt at transplant (g)	Blood sugar at transplant (mmol/l)		Wt 2wks post transplant	Blood sugar 2wks post transplant	Wt 4wks post transplant	Blood sugar 4wks post transplant
Male	233	27		266	10.4		
Male	234	21.8		248	13.2		
Male	206	33.3		223	24.5		
Female	175	33.3		174	9.8	195	5.5
Female	163	28.3		163	16.1	180	21.6
Female	174	33.3		181	11.6		
Female	176	32		200	5.3		
Female	181	33.3		184	5.7		

3.2.5 Validation of sex mismatched right branch intra-portal transplantation

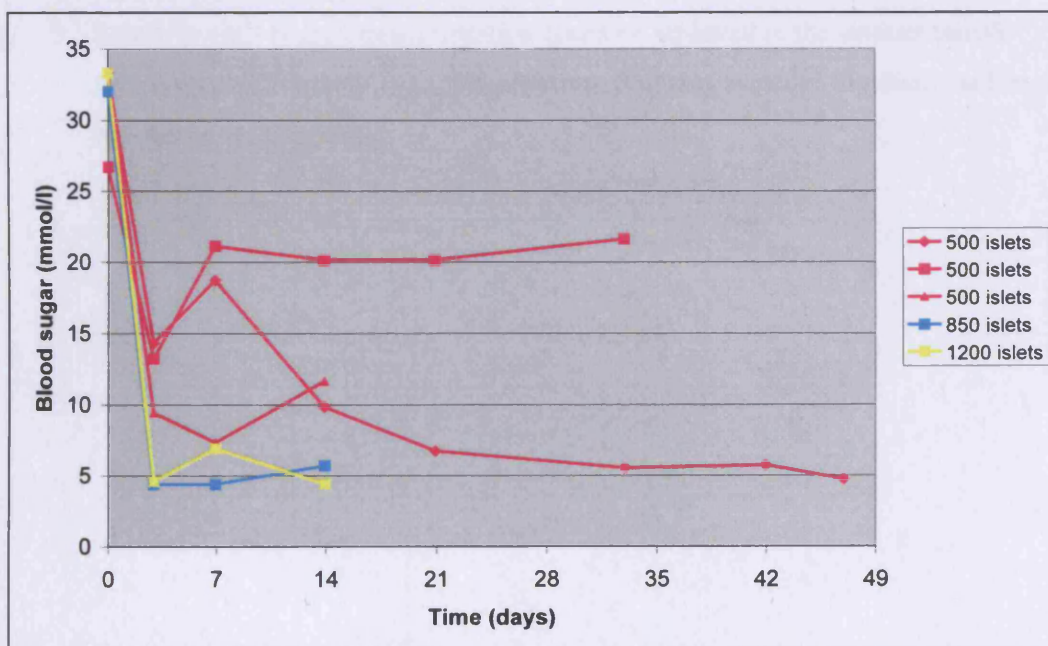
With the success of the non selective male to female transplants, the next experiments were designed to investigate whether selective transplantation was feasible in the smaller female recipients, the dose of islets required to reverse STZ induced diabetes and whether a longer post-transplant course affected outcomes.

Five female recipients were rendered diabetic with streptozotocin and transplanted with male islets. Three of the recipients received a dose of 500 islets, one 850 islets and one

1200 islets. One recipient from each group was sacrificed after 14 days, the original planned time for the right branch experiments, and a further two animals in the 500 islet group were sacrificed after five and seven weeks, in order to investigate any effect of sex mismatching on long term graft outcome.

The average weights of the animals fell from 195.2g (\pm 6.8) pre streptozotocin to 173.4g (\pm 6.6) on the day of transplant and then rose to 181.6g (\pm 11.5) fourteen days after transplantation. The change in blood sugars post transplant is illustrated in figure 3.2.

Figure 3.2: Change in blood glucose after transplant in 5 diabetic female recipients receiving male islets transplanted to the main branch portal vein.



With the success of the sex mismatched main branch transplants, the experiments were next performed with right branch transplantation.

3.2.6 Conclusions of in-vivo validation

These experiments proved that:

1. Measurement of rat tail vein blood glucose with a hand held glucometer provided a good estimation of glucose concentration which was comparable to the gold standard but less traumatic to animals.
2. Stable induction of diabetes could be achieved with i.p. injection of STZ at a dose of 55mg/Kg in male rats and 50mg/Kg in female rats.
3. Isolation of islets was reproducible and optimised when using 3.3ml of buffer for injection of collagenase and 1.084g/ml ficoll density for purification
4. Diabetes could be reversed with the intra-portal transplantation of islets and male to female islet transplantation did not obviously affect the outcome in a syngeneic transplant model.
5. Selective right branch transplantation could be achieved in the smaller female recipients and a dose of over 500 islets was probably required to reliably achieve normalisation of glucose.

3.3 Development of histological techniques

The histological examination of transplanted islets was an important end point for the analysis of engraftment. The large volume of histology sections, cost of sample processing and need for optimal staining necessitated a clear understanding of the processes of tissue handling and processing. The basic techniques were learned within the routine clinical histopathology service and further optimised for islet staining and graft analysis.

3.3.1 Tissue sectioning and mounting

The analysis of islet grafts required retrieval of recipient livers, fixation, sectioning and staining. The fixation was performed in 4% formaldehyde for short periods, in order to reduce the antigen retrieval required for subsequent staining. The sectioning of paraffin embedded livers proved difficult due to fracturing and crumpling of tissue, caused by the short retrieval time and nature of liver tissue. This required modification of the sectioning technique and, after some experimentation, it was found that wetting the tissue prior to cooling tissue blocks and cutting at an incident angle below 10° reduced damage to tissue.

Once good tissue sectioning was achieved, the tissue had to be mounted on coated slides. The harsh processing required for immunohistochemistry and fluorescence often lead to the loss of tissue from slides and uncoated slides did not provide sufficient tissue adhesion. The slides were initially coated with Poly-l-lysine (Sigma Aldrich, UK), to improve adhesion, but tissue was still lost during processing. A combination of Poly-l-lysine and baking of slides at 65°C provided excellent tissue adhesion but unfortunately caused loss of the antigen necessary for the binding of *B. Simplicifolia*. The slide coating was changed to Vectabond (Vector Laboratories, UK) and tissue adhesion was found to be sufficient without the need for baking. Therefore, Vectabond coated slides were used with air drying for improving tissue adhesion.

3.3.2 Histology assessment of intra-portal transplanted islets.

The examination of engrafting islets during the process of revascularisation required a robust and quantifiable method for imaging both the endocrine mass and the vascular endothelium within transplanted islets.

Initial histological analysis was undertaken with haematoxylin and eosin staining (figure 3.3 A). This basic staining provided a reasonable delineation between liver and transplanted islet tissue which could be used for analysing graft area but there was no clear delineating of endocrine area and endothelium was not clearly identified. Therefore, immunohistochemical techniques were used to analyse the different composition of the islets.

Sections were stained for insulin by immunohistochemistry (figure 3.3 B) which provided good visualisation of the β -cell mass of islets. Unfortunately, the endothelial markers Factor VIII related antigen, CD31 and CD34 (figure 3.3 C, D and E, respectively) were limited by background peroxidase staining and inability to demarcate islet endothelium sufficiently.

Due to the difficulties of reliably identifying the transplanted islet endothelium, the endothelial marker was changed to the lectin *Bandeiraea Simplicifolia*(BS-1). This lectin binds to endothelial carbohydrates and is a more reliable marker of transplanted islet endothelium than CD31, CD34 or von Willebrand factor (Mattsson, Carlsson et al. 2002). In addition, the staining technique was changed from immunohistochemistry to immunofluorescence, in order to enable two colour staining for the simultaneous analysis of islet endothelium and β -cell mass without the need for multiple enzymatic incubations.

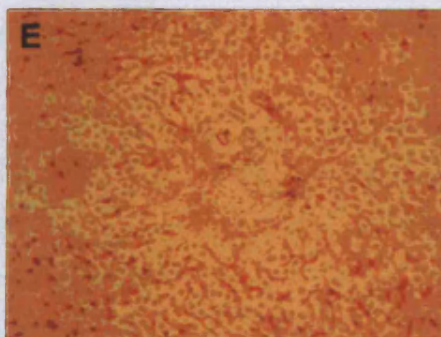
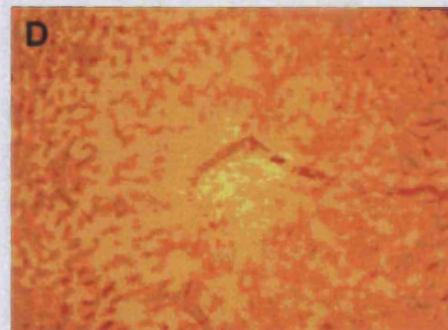
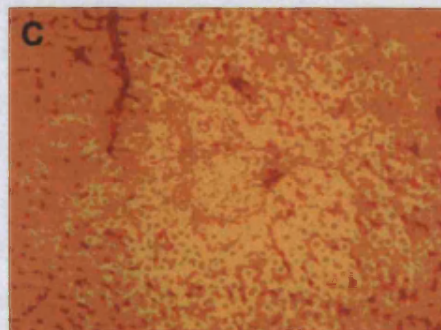
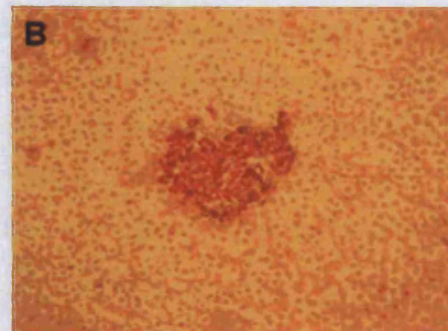
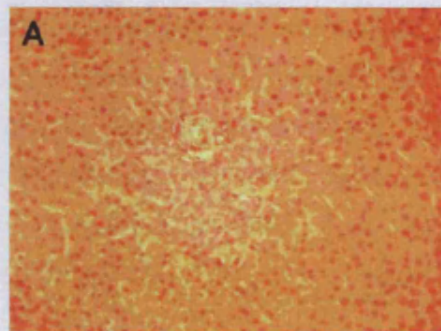


Figure 3.3: Serial sections of recipient rat liver (x 200) stained with:

- A Haematoxylin and Eosin**
- B Insulin**
- C CD 34**
- D CD31**
- E Factor VIII**

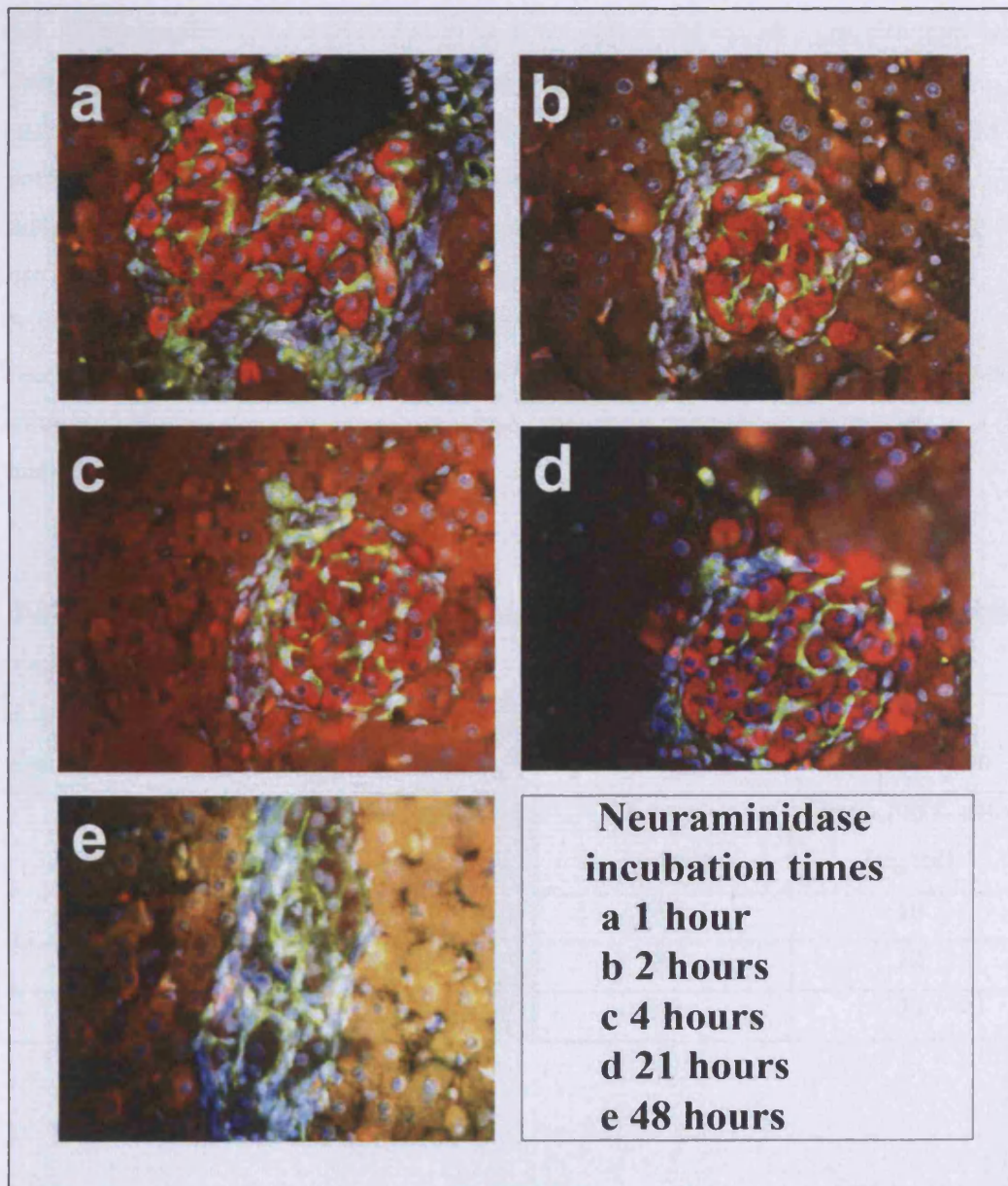
Initial results with *B. Simplicifolia* staining did not achieve satisfactory delineation of the vascular endothelium. Multiple antigen retrieval techniques (including trypsin digestion, heat treatment in citrate buffer and heat treatment in Tris-EDTA buffer) were used without any improvement in staining. Therefore, the *B. Simplicifolia* - FITC staining complex was changed to biotinylated *B. Simplicifolia* with a secondary layer of streptavidin FITC, in order to provide amplification of signal and improve visualisation. Unfortunately, there was still no improvement in resolution.

After discussion with Goran Mattson (Mattsson, Carlsson et al. 2002), it became clear that he used antigen retrieval with neuraminidase 0.1u/ml in 0.1M sodium acetate, which was not highlighted in his original publication. Subsequent experiments using antigen retrieval with neuraminidase brought about the staining of islet endothelium with *B. Simplicifolia* but appeared to be variable, with some experiments staining and others not staining at all. After further experimentation, it was found that the slide baking step (incubation of slides at 65°C overnight) introduced to improve tissue adhesion to slides interfered with BS-1 staining. This issue was resolved by changing the adhesive coating of slides to Vectabond and avoiding the baking of slides by air drying.

The antigen retrieval of insulin with neuraminidase worked well but longer incubations appeared to cause some degradation of insulin signal. Therefore, a further experiment was undertaken to ascertain the optimum incubation time for tissue pre-treatment with neuraminidase (figure 3.4).

This experiment showed that an incubation time of ~21 hours was found to be optimal for both retrieval of insulin and BS-1. Shorter incubation times (1, 2 and 4 hours) produced patchy BS-1 staining with good insulin retrieval but longer incubation times (48 hours incubation) produced no insulin staining.

Figure 3.4: Neuraminidase antigen retrieval optimisation. Images are of rat liver transplanted with islets (x 200). The sections have been stained simultaneously with *B. Simplicifolia* (green), insulin (red) and counterstained with DAPI (blue). The antigens have been retrieved with neuraminidase applied for differing periods of time.



A further experiment was performed on neuraminidase treated tissue to optimise concentrations of biotinylated BS-1, FITC-streptavidin, mouse anti-insulin antibody and rhodamine-conjugated anti-mouse antibody. The first experiments used three different concentrations of BS-1 (10µg/ml, 20µg/ml and 30µg/ml) and three different concentrations of FITC-Streptavidin (10µg/ml, 20µg/ml and 30µg/ml), as set out in table 3.3. All the combinations produced staining of transplant islet endothelium, although the staining with 10µg/ml concentrations was patchy. A similar set of experiments were undertaken for insulin staining with three different concentrations of mouse anti-insulin antibody (1:1000, 1:500 and 1:100) and rhodamine conjugated sheep anti-mouse antibody (1:1000, 1:500 and 1:100). Although both antibodies were recommended for use at a concentration of 1:1000 by the manufacturer, the optimal concentrations for both antibodies was found to be 1:500. Therefore, the optimal dilutions needed to produce consistent staining were 20µg/ml of both BS-1 and streptavidin-FITC and 1:500 dilutions of both mouse anti-insulin and rhodamine-conjugated sheep anti-mouse antibodies.

Table 3.3: Experimental design for optimising the concentration of biotinylated BS-1 and streptavidin-FITC.

Experiment 1: Optimising BS-1 concentration		Experiment 2: Optimising strep-FITC concentration	
BS-1 conc (µg/ml)	Strep. FITC conc (µg/ml)	BS-1 conc (µg/ml)	Strep. FITC conc (µg/ml)
10	30	30	10
20	30	30	20
30	30	30	30

3.3.3 Conclusions of histological optimisation

These experiments proved that:

1. Tissue sections should be air dried and mounted on Vectabond coated slides to avoid tissue loss and poor staining with BS-1.
2. The immunohistochemistry techniques tested were not suitable for dual staining of endothelium and insulin in transplanted islets.
3. Dual fluorescent BS-1 and insulin staining provided good delineation of endothelium and β -cell mass in transplanted islets.
4. The optimal protocol for dual staining was:
 - a. 21 hours antigen retrieval with neuramidase.
 - b. 20 μ g/ml of both BS-1 and streptavidin-FITC.
 - c. 1:500 dilutions for both mouse anti-insulin and rhodamine anti-mouse antibodies.

3.4 Development of in-vitro assays for growth factor protein expression

The initial aim was to assess the effect of desferrioxamine treatment on growth factor protein expression. This section describes the optimisation of protein assays and problems encountered when analysing growth factor protein expression.

3.4.1 Assay of VEGF protein expression

The analysis of VEGF in culture supernatants was straight forward and did not require dilution of the culture media. The cross reactivity of the mouse VEGF kit was well established and ELISA plates developed well.

3.4.2 Assay of bFGF protein expression

With the success of the VEGF protein estimation, the culture supernatants were analysed for other important angiogenic factors. Unfortunately, rat or mouse-specific ELISA's were not available for bFGF and HGF but a number of authors had reported the use of human-specific ELISA kits from R&D to assay rat bFGF (Kobayashi, Hamano et al. 2000; Li, Khosla et al. 2000; Neuner-Jehle, Berghe et al. 2000) and the manufacturers suggested that the kits were sufficiently cross reactive with rat bFGF. Therefore, the human specific ELISA for bFGF (R&D Systems) was used to analyse rat bFGF levels in culture supernatants.

The initial results were positive and suggested that desferrioxamine treatment up regulated bFGF (figure 3.5) but the values were variable, with development of signal in only a few ELISA plate wells (table 3.5). These results did not provide reliable evidence of bFGF expression and it was initially thought that the poor signal was due to the concentration of bFGF in supernatants being below the sensitivity of the assay. The experiments were repeated with 100 and then 200 islets per well but the increase in islet number did not improve the signal. In order to rule out the effect of freeze thaw cycles, the experiments were repeated with fresh supernatants from cultured islets, but there was still no signal. Finally, experiments were performed using 350 islets in culture with concentration of the supernatant 10 fold using a Microcon YM-3 centrifugal filter unit

(Millipore, Watford, UK). This assay was run in parallel with lysates from cultured islets. The experiment provided a good signal from lysed and concentrated samples with estimated concentrations of bFGF in culture supernatants of 2.3pg/ml and 2.9pg/ml for the DFO 100 and control cultures, respectively. The bFGF signal from lysed islets was better and the assay involved fewer steps than that from concentrated supernatants. Therefore, the technique of islet lysis was used for the further assays of bFGF protein estimation.

Figure 3.5: Mean bFGF concentration in supernatants from islet cultures after overnight incubation in increasing concentrations of desferrioxamine (DFO 10 =desferrioxamine 10 μ M, DFO 100 = desferrioxamine 100 μ M and DFO 1000 = desferrioxamine 1000 μ M). Four sets of cultures were used in each group.

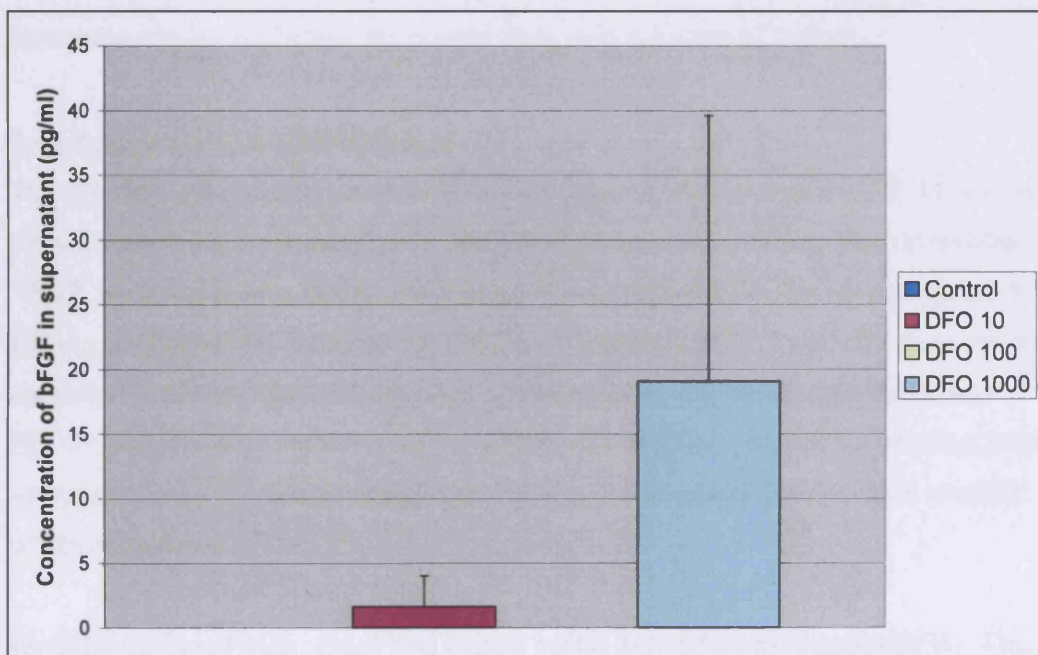


Table 3.4: ELISA plate readings of bFGF concentration (pg/ml) in supernatants from islet cultures after overnight incubation in increasing concentrations of desferrioxamine.

Experiment	Control	DFO 10	DFO 100	DFO1000
1	0	0	0	46.94
2	0	1.5	0	0
3	0	5.02	0	21.18
4	0	0	0	8.22

3.4.3 Assay of HGF protein expression

A human HGF ELISA (R&D Systems) did not indicate evidence of HGF within the culture media, despite increasing the dose of cultured islets. In view of the mRNA expression results, see below, the protein assay was not pursued further.

3.4.4 Assay of HIF-1 α concentrations

The hypothesis for treating islets with desferrioxamine is to up regulate HIF-1 α and the down stream hypoxia response molecules. Desferrioxamine treatment does up regulate VEGF, as shown below, but the mechanism is only presumed unless the up regulation of HIF-1 α can be proven. An assay for HIF-1 α was required at the protein level, as the up regulation is due to reduced destruction by proteosomes and occurs post translation. HIF-1 α protein assays are not readily available and therefore, the duo set developmental sandwich ELISA for mouse/human total HIF-1 α (R&D systems) was tried to quantify protein expression.

Overnight cultured islets were lysed in lysis buffer #11 and assayed immediately. The experiments with 100, 200 and 350 islets or the supernatant from a 350 islet culture did not give a signal. It was presumed that the assay was either not sufficiently specific for rat HIF-1 α , despite the highly conserved structure between species, or the level of HIF-1 α in the samples was below the sensitivity of the assay. The escalation of islet quantity was not justifiable, given the animal source of the islets, and it was decided to use the assay with desferrioxamine treated human islets, where a high dose of islets could be

used and the specificity of the assay would be correct. Unfortunately, there were no suitable islet preparations available after this time and there was insufficient time left to establish a western blot analysis technique for rat HIF.

3.4.5 Conclusions from in-vitro assay of growth factor protein expression

These experiments proved that:

1. Vascular endothelial growth factor protein expression can be easily analysed in islet culture supernatants.
2. Basic fibroblast growth factor should be analysed from lysed cells.
3. Hepatocyte growth factor and HIF-1 α ELISAs are not sufficiently sensitive or cross reactive to analyse the protein expression of these factors in cell culture supernatants.

3.5 Development of molecular techniques

Although molecular methods were used within the laboratory, they had not been applied to the study of islets before. This section will examine the reasoning for choosing specific methods and the work up of the primer sets.

3.5.1 Isolation of RNA

Ribonucleic acid (RNA) is usually separated from DNA and cellular proteins using its specific physico-chemical properties by phenol chloroform extraction. This technique was originally described by Chomczynski and Sacchi in 1987 and a variety of simplified methods have been designed since the publication of the original method (Chomczynski and Sacchi 1987) .

RNA was initially isolated in accordance with the original Chomczynski and Sacchi protocol but the methodology was time consuming and the purity and quality of RNA was poor (See table 3.5). The ratio of absorbance of 280nm to 260nm was often low (values 0.9-1.2) and samples of isolated RNA frequently amplified by PCR, suggesting unacceptable DNA contamination. The RNA isolation methodology was therefore changed to a GenElute mammalian RNA isolation kit (Sigma Aldrich, UK), which is based on a silica gel system for purification. This technique reduced the time required for purification of RNA and increased yield but did not improve the purity. Therefore, the isolation technique was changed back to a phenol/chloroform based isolation technique (TRIzol reagent from Invitrogen) but used a premixed guanidium/phenol mixture. The change in technique improved RNA purity, although the yield was not as good as the silica gel based technique.

When the RNA extraction of fresh islets was compared to cultured islets, it appeared that RNA purity and content were much better in fresh islets (table 3.6). Although a number of the steps involved in RNA extraction were varied, the RNA ratio could not be improved in the cultured islets. It was unclear whether this observation was related to culture but after changing the chloroform to fresh stock, the ratio was improved when isolating RNA from hypoxic islets.

Table 3.5: Analysis of different RNA purification methods. Each isolation was tested by using overnight cultured islets isolated from a single rat. RNA was diluted in a final volume of 50 μ l. Values are expressed as mean and standard error.

	Chomczynski and Sacchi method	GenElute (Sigma Aldrich)	TRIzol reagent (Invitrogen)
Number of isolations	5	4	4
RNA conc. (μg/ml)	78.9 (\pm 37.5)	188.6 (\pm 38.1)	122.2 (\pm 15.1)
Ratio	1.039 (\pm 0.06)	1.16 (\pm 0.04)	1.50 (\pm 0.06)
Protein conc. (mg/μl)	3.32 (\pm 0.31)	2.75 (\pm 0.48)	0.75 (\pm 0.15)

Table 3.6: Analysis of RNA isolated from fresh, control cultured, desferrioxamine treated and hypoxic cultured islets. RNA was diluted in a final volume of 50 μ l. Values expressed as mean and standard error.

	Fresh islets	Control culture	DFO 100	DFO 1000	Hypoxic
RNA conc. (μg/ml)	482.2 (91.7)	108.9 (25.4)	102.6 (30.1)	134 (51.3)	490.8 (64.5)
Ratio	1.70 (0.02)	1.60 (0.03)	1.55 (0.03)	1.55 (0.03)	1.73 (0.02)
Protein conc. (mg/μl)	1.83 (0.25)	0.5 (0.18)	0.63 (0.21)	0.75 (0.22)	0.46 (0.07)
Purity	93.3 (1.13)	88.3 (1.71)	86 (1.78)	86 (1.74)	95.8 (1.4)

3.5.2 Additional primer sets.

A number of primer pairs were evaluated for examining the expression of key angiogenic factors (table 3.7) before selecting the final primer pairs, as described in chapter 2. All primer pairs were selected in accordance with the methodology outlined in chapter 2

Table 3.7: Primer pairs evaluated but not suitable for final experiments. Figures in brackets refer to additional primer pairs analysed, not different molecules.

Growth factor	Forward primer	Reverse primer
bFGF (1)	AGTTGTGTCCATCAAGGGAGTGT	TGGACTCCAGGCGTTCAAAG
bFGF (2)	GGCTTCTAAGTGTGTTACAGAAGAGTGTT	GAGTATTTCCGTGACCGGTAAGTG
bFGF (4)	GCTGGCTTCTAAGTGTGTTACAGAAGAGT	CAGTGCCACATACCAACTGGAGTA
HGF	GTGTCAGCGTTGGGATTCG	TTGAAGTTCTCGGGAGTGATGATGTC
VEGF	AATGCAGACCAAAGAAAGATAGAACA	CGCTCTGAACAAGGCTCACA
ANG1 (1)	GCCTGTGGCCCTTCCAAT	TGGTTTTGCCCTGCACTGTA
ANG1 (2)	TAACAGGAGGTTGGTGGTTTGA	TTTGCCCTGCAGTGTAAGAACA
ANG1 (4)	TAACAGGAGGTTGGTGGTTTGA	TGGTTTTGCCCTGCAGTGTA
Endostatin	CCTCTAGGCTGCAGGACCTCTA	GGTTGACGATGGGCACAGA
18S	CCTGAGAAACGGCTACCACATC	GGGTCGGGAGTGGGTAATTT
GAPDH(1)	TGCCAAGTATGATGACATCAAGAAG	GTAGCCCAGGATGCCCTTTAG
GAPDH(2)	GAGAAACCTGCCAAGTATGATGACA	AGCCCAGGATGCCCTTTAGT

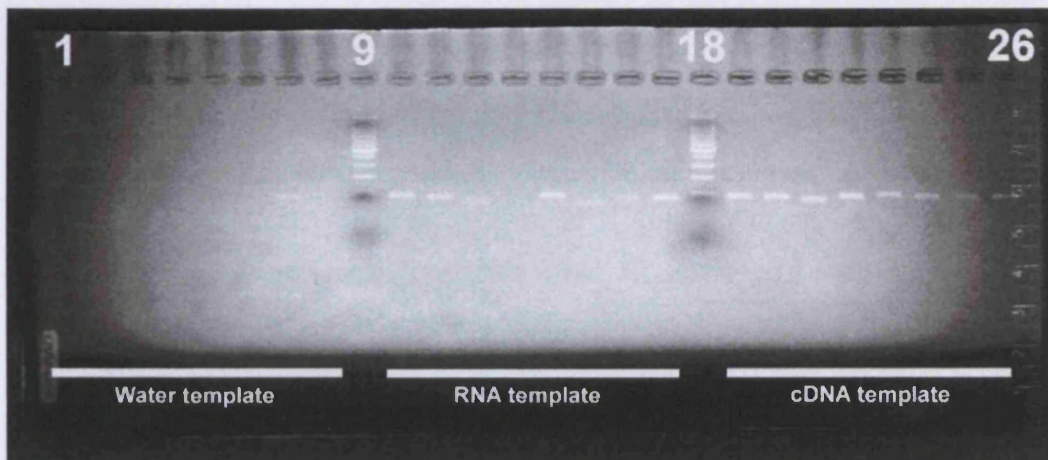
3.5.3 Formation of cDNA

Two different methodologies were evaluated for the formation of cDNA. The first was a bead technique using “Ready-to-go you-prime first-strand beads” (Amersham Biosciences, Buckinghamshire, UK). This technique involved incubating 2µg of total RNA and 0.2µg of random hexamers (Amersham Biosciences, Buckinghamshire, UK) in 30µl of water, with two beads containing dNTPs, reverse transcriptase and RNase inhibitors. The reaction was incubated for 1 hour and subsequent cDNA used for both standard PCR and real time PCR. Although this technique did produce amplifiable cDNA, the real time amplification was poor and produced distorted amplification curves that were unacceptable for real time PCR analysis. The Taq-Man reverse transcription reagents (Applied Biosystems, Warrington, UK) were evaluated and found to provide cDNA which amplified well, in a reproducible fashion. This method was used for subsequent cDNA formation.

3.5.4 DNA contamination

Contamination of real time PCR reactions with DNA is an important issue, as any amplification in addition to cDNA will lead to an increase in SYBR green signal and distortion of the relative quantification of gene expression. The source of DNA contamination is two fold. Firstly, DNA contamination could be exogenous from external contamination (i.e. from poor handling, contamination of water and reagents, contamination of plastic ware and contamination of work environment etc). Secondly, contamination could be endogenous (i.e. genomic DNA contamination of the RNA). During the evaluation of primer pairs, both types of contamination were identified, figure 3.6

Figure 3.6: Gel electrophoresis of PCR products using primers (from left to right GAPDH, beta actin, VEGF all, VEGF 164, bFGF, HGF, ANG1 and Endostatin). Lane 1-8 are water controls, 10-17 have RNA only as a template and 19-27 have cDNA as template. Lane 9 and 18 are 100bp ladders. Exogenous DNA contamination can be seen by amplification of the water control lane 7 and endogenous contamination is seen by amplification of the RNA lanes 10-12 and 14-17.



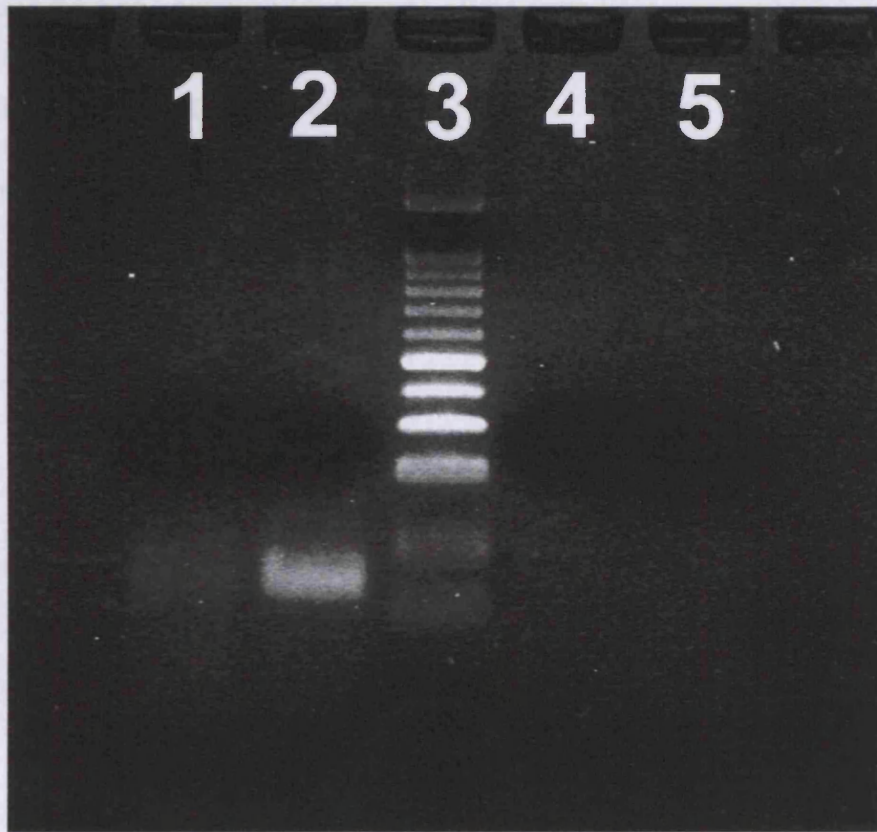
3.5.4a Reduction of exogenous DNA contamination

The amplification of water controls, as above, suggested exogenous DNA contamination of the PCR reactions. The source of the contamination was probably a combination of the water used for diluting reactions, plastic ware and handling of samples. The water used for RNA and PCR reaction dilution was initially double distilled water which had been DEPC treated and autoclaved. It became clear that this was a major source of contamination and was probably due to the use of laboratory glassware which had been contaminated with rat genomic DNA whilst being used for buffers during other rat experiments. Therefore, the water source was changed to “water for molecular biology” (Sigma Aldrich, UK), which was certified DNA, DNase and RNase free, and was stored in fresh plastic tubes. All plastic ware was changed and only unopened bags were used with no handling, other than with clean forceps. In addition, all primers and reagents were replaced and the place of experimentation was changed to a clean area where no rat experiments were performed.

3.5.4b Reduction of endogenous DNA contamination

Genomic DNA contamination of RNA samples was evident by the amplification of RNA samples by standard PCR, as above. A number of different RNA extraction techniques had already been tried to reduce contamination but ratios of 1.6-1.7 were still achieved. The issue was whether to reduce DNA contamination of RNA or increase the specificity of the PCR primers for cDNA, so that only cDNA was amplified and not genomic DNA. Both techniques were employed and the improved specificity of PCR primers is described later in this chapter. The reduction of genomic DNA contamination in RNA samples was achieved by adding a DNase step to the RNA extraction protocol, before formation of cDNA. This additional step reduced DNA contamination and PCR amplification of RNA, as shown in figure 3.7.

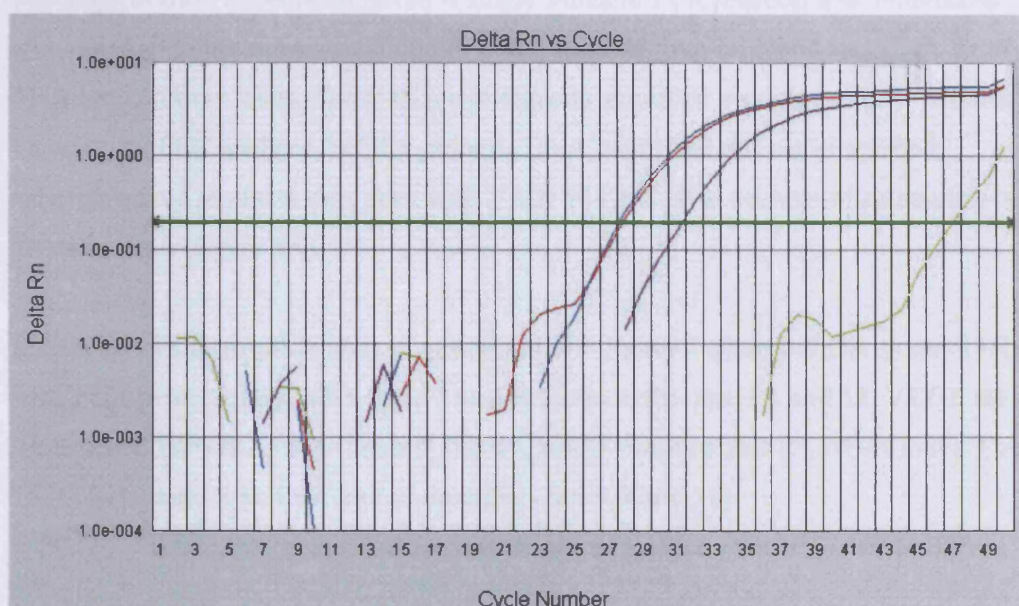
Figure 3.7: Reduction of genomic DNA contamination by DNase treatment of RNA. The image shows a gel electrophoresis of standard PCR products amplified with beta actin primers. Lane 1 is RNA treated with DNase, lane 2 is non treated RNA, lane 3 is a 100bp ladder and lane 4 and 5 are water controls.



After the reduction in DNA contamination with DNase, the next step was to show that DNase treatment of the RNA sample did not interfere with cDNA formation, concentration or amplification. Real time PCR was used to examine the effect of DNase treatment by running parallel real time PCR amplifications of RNA versus DNase treated RNA versus cDNA formed using untreated RNA versus cDNA formed using DNase treated RNA. The same original RNA sample was used for each parallel PCR amplification and all the dilutions were

carefully calculated so that the same concentration of original isolated RNA was used in each reaction, therefore allowing direct comparison of all groups.

Figure 3.8: Real time PCR amplification with HGF primers using cDNA from untreated RNA as a template (red curve), cDNA from DNase treated RNA as a template (blue curve), untreated RNA as a template (purple curve) and DNase treated RNA as a template (green curve).



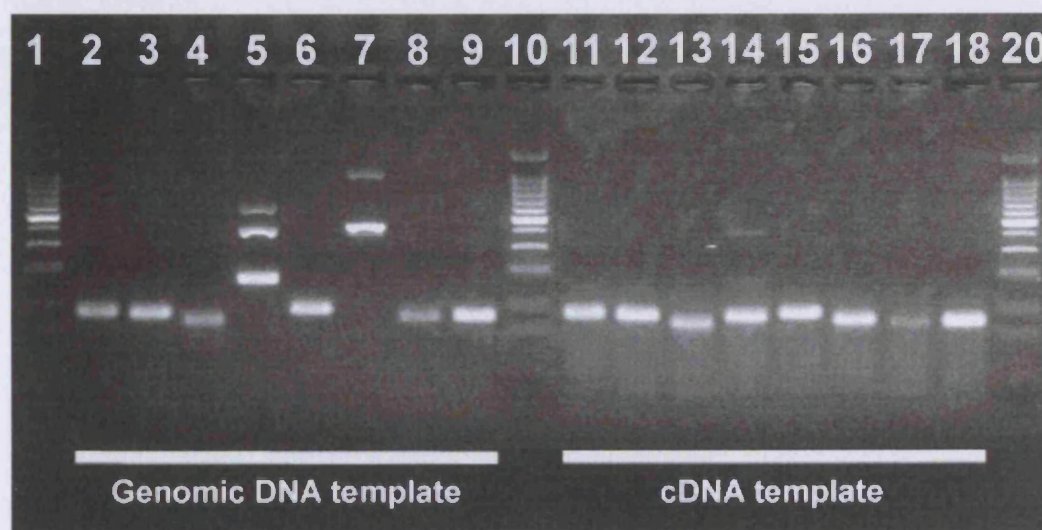
This experiment showed that DNase treatment reduced genomic DNA contamination of RNA without effecting the concentration or amplification of cDNA (figure 3.8). In addition, even without DNase treatment, the amplification of genomic DNA in the RNA sample was less than the amplification of cDNA (figure 3.8). When calculated from this example, the genomic DNA amplified 16 fold less than the cDNA.

3.5.5 Evaluation of primers

Due to the problems with genomic DNA amplification, the primer sets were redesigned to try to improve the specificity of binding and stop the amplification of genomic DNA. The redesigned primers were reevaluated for their efficacy, specificity and amplification kinetics.

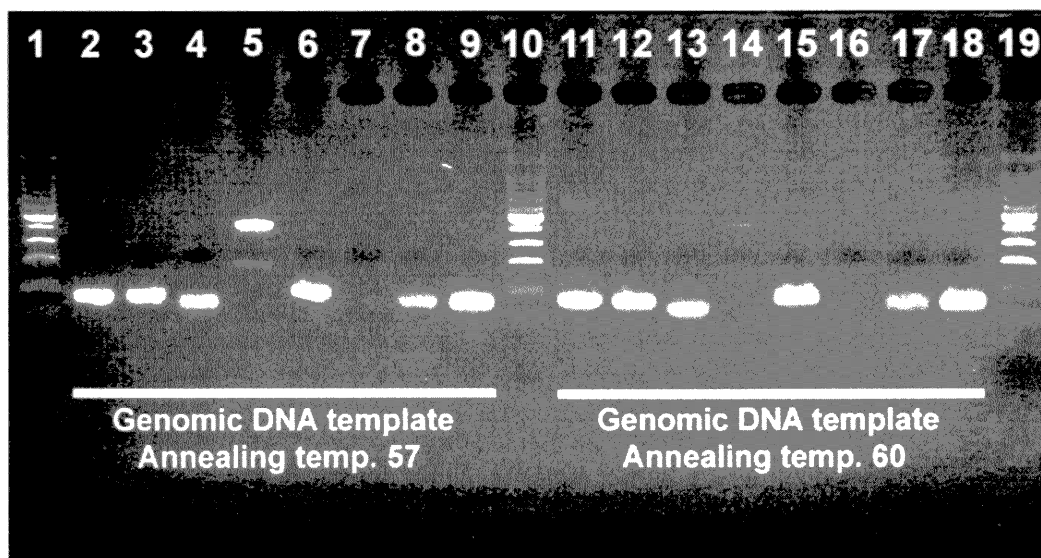
The first step was to ensure that the primers amplified cDNA and evaluate whether there was amplification of genomic DNA. A single standard PCR reaction was undertaken with the primer sets and either genomic DNA, isolated from rat blood, or cDNA from RNA isolated from islets. The cDNA experiments amplified a single product, important for real time PCR analysis, but the genomic DNA amplifications also amplified; although mixed products were seen with VEGF164 and HGF primers when amplifying genomic DNA (figure 3.9)

Figure 3.9: PCR amplification of genomic DNA (lanes 2-9) and cDNA (lanes 11-18) with PCR primers (GAPDH- lane 2 and 11, beta actin- lanes 3 and 12, VEGF all- lanes 4 and 13, VEGF 164- lanes 5 and 14, bFGF- lanes 6 and 15, HGF- lanes 7 and 16, ANG1- lanes 8 and 17, and endostatin - lanes 9 and 18).



Following on from this experiment, the conditions of the PCR reaction were altered to reduce amplification of genomic DNA. The first step was to increase the annealing temperature of the PCR cycle to a point closer to the melting temperature of the primers. This change to the PCR cycle should make the primers more specific for cDNA, due to the overlap of one primer across an exon-exon boundary, and avoid amplification of genomic DNA. These experiments were performed with genomic DNA as a template and annealing temperatures of 57°C and 60 °C. The increased annealing temperatures made no difference to the amplification of genomic DNA with primers, except HGF and VEGF164 where amplification was reduced or abolished (figure 3.10).

Figure 3.10: PCR amplification of genomic DNA with different annealing temperatures, 57°C and 60 °C. Lanes 1, 10 and 19 are 100bp ladder. The primer pairs used in the remaining lanes are: GAPDH- lanes 2 and 11, beta actin- lanes 3 and 12, VEGF all- lanes 4 and 13, VEGF 164- lanes 5 and 14, bFGF- lanes 6 and 15, HGF- lanes 7 and 16, ANG1- lanes 8 and 17, and endostatin- lanes 9 and 18.



These primer pairs were used for the collection of gene expression data, as outlined in chapter 4. All primers worked well during real time PCR with no amplification of DNase treated RNA, under the predetermined PCR cycle conditions. Unfortunately, the bFGF, Ang1 and endostatin primers showed amplification in the control RNA samples. Therefore, this data was discarded and the primers for these growth factors were redesigned.

The genomic structure and mRNA sequence of bFGF, Ang-1 and endostatin were re-scrutinised and further primer sets were designed. Two bFGF primer pairs were redesigned and reordered while a single primer pair was reordered for Ang1 and a further primer pair created from the forward primer of the first Ang1 pair and the reverse primer from the second Ang1 primers. The new primer pairs were further evaluated and optimized.

Initial experiments with the new primers gave similar results (figure 3.11) but further optimization with increased annealing temperature to 63.5°C and addition of deionised formamide to a final concentration of 2%, increased the specificity of primer binding and stopped genomic DNA amplification of the new primers (figure 3.12). Unfortunately, the addition of deionised formamide reduced the efficiency of the beta actin primers and further control amplifications with beta actin were undertaken without the addition of formamide. These new primer sets were used to rerun the real time PCR evaluation of bFGF, Ang1 and endostatin gene expression in the experimental samples.

Figure 3.11: Electrophoresis gel of PCR products from amplification of RNA (lane 2-6) and cDNA (lane 8-12) using redesigned Ang1 and bFGF primers. Lanes 1 and 7 are 100bp ladder. The other lanes are with the following primers: Beta actin- lane 2 and 8, bFGF (3)- lanes 3 and 9, bFGF (4)- lanes 4 and 10, Ang1 (3)- lanes 5 and 11, Ang1 (4)- lanes 6 and 12.

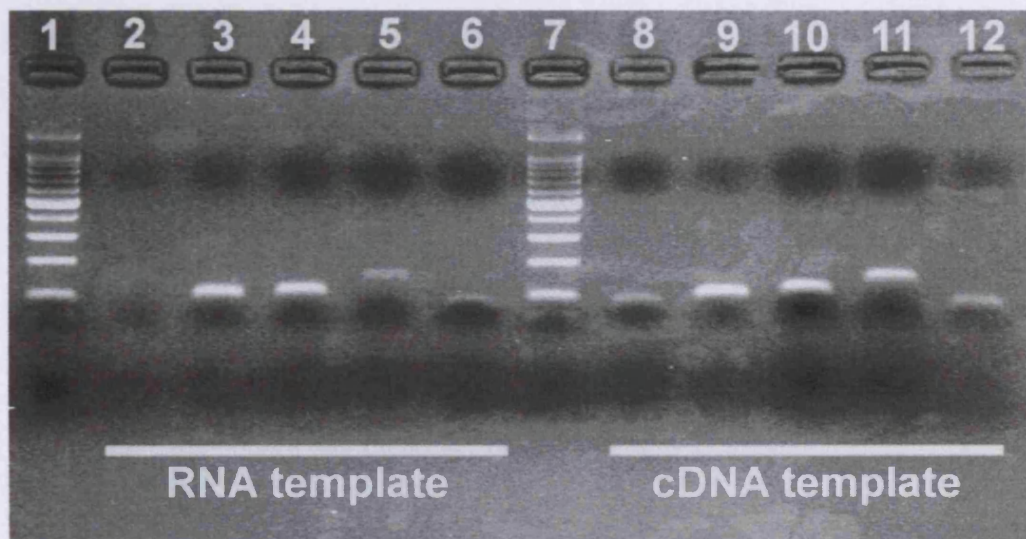
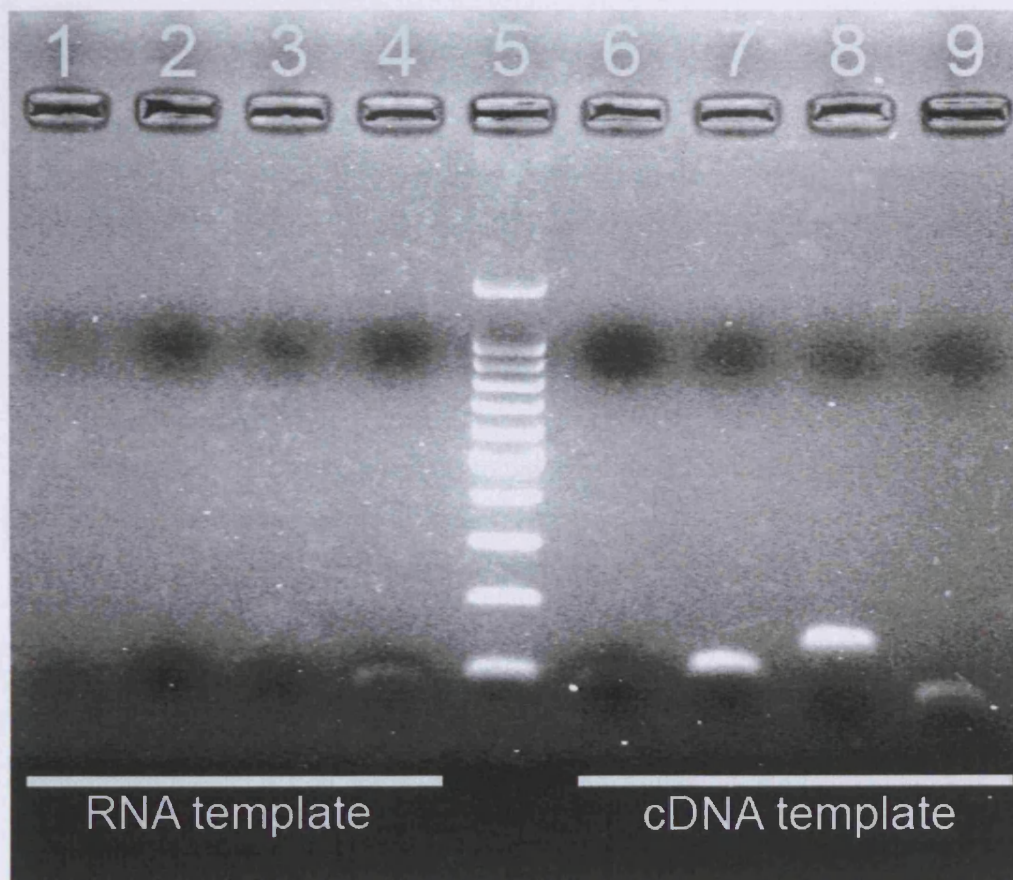


Figure 3.12: Electrophoresis gel of PCR products from amplification of RNA template (lanes 1-4) and cDNA template (lanes 6-9), in the presence of 2% deionised formamide and using annealing temperature of 63.5°C. The following primers were used: beta actin- lanes 1 and 6, bFGF (3)- lanes 2 and 7, Ang1 (3)- lanes 3 and 8, endostatin- lanes 4 and 9.



3.5.6 Selection of a control primer pair

The use of real time PCR for quantifying the relative expression of target genes relies on a suitable control (or housekeeping) gene whose expression is not altered by the experimental conditions. Three candidate control genes were selected, beta-actin, GAPDH and 18S. All three genes had been used before by other authors in similar experiments and all genes were evaluated for use as controls.

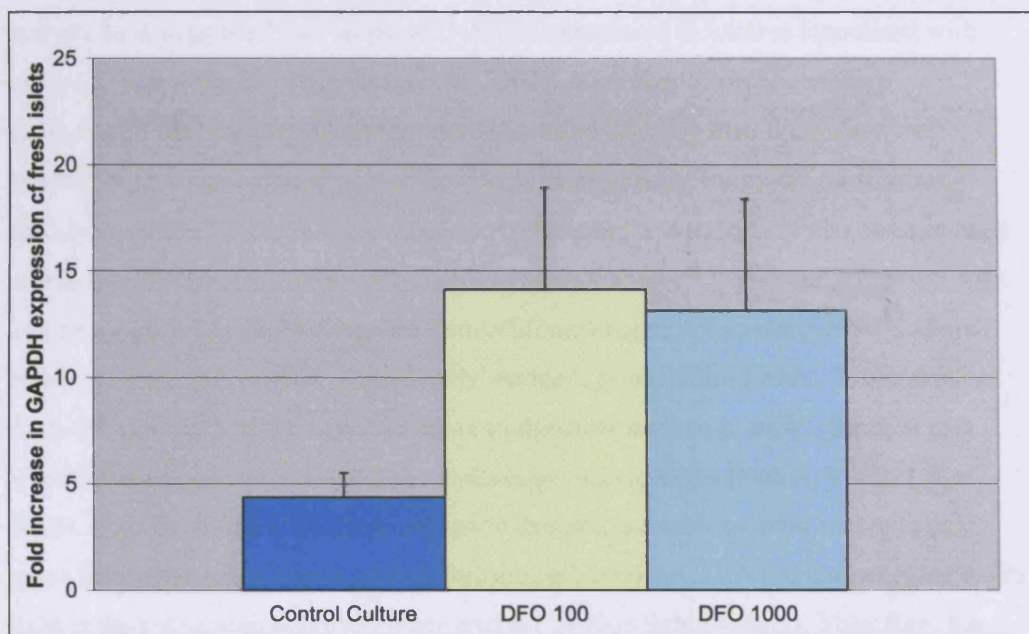
The 18S primer pairs were discarded first. Despite two different primer pairs being evaluated, the 18S primers amplified genomic DNA and the amplification curves produced by real time PCR had different kinetics to the primers for other target genes.

The second primer pair to be discarded was GAPDH. Despite having good amplification curves for real time PCR, the expression was altered under differing experimental conditions when compared to the other two control primers. This is probably because GAPDH is up-regulated by hypoxia (Escoubet, Planes et al. 1999; Zhong and Simons 1999) and hypoxia inducible factor (Graven, Bellur et al. 2003). The up-regulation of GAPDH by hypoxia and HIF is consistent with our observations, where GAPDH gene expression was up-regulated by iron depletion; figure 3.13

Beta actin was also compared to 18S expression in a small number of amplifications where reasonable 18S curves could be achieved and Ct was evaluated at the threshold point. These two experiments showed there was no significant change in the ΔC_t between beta actin and 18S when islets were treated with desferrioxamine. (cc ΔC_t -9.32 (0.28), DFO 100 ΔC_t -9.62 (0.53), DFO 1000 ΔC_t -9.43 (0.37))

Therefore, these experiments suggested that there was a significant up regulation of GAPDH in response to desferrioxamine treatment but 18S and beta actin were probably unchanged. Beta actin was subsequently used as the control (housekeeping) gene, as expression was stable under iron depletion, the primer pairs did not preferentially amplify genomic DNA and the amplification kinetics were similar to the experimental target genes.

Figure 3.13: Relative change in GAPDH expression in control culture and desferrioxamine treated islets when compared to fresh islets, calculated by using beta actin as control. All changes in expression were significant at the level $p < 0.01$.



3.5.7 Conclusion from the development of molecular techniques

These experiments proved that:

1. TRIzol was the most effective and reproducible method for isolating RNA from islets.
2. Exogenous DNA contamination could be eliminated by careful handling and use of certified DNA free consumables and solutions.
3. Endogenous DNA contamination could be eliminated by:
 - a. Addition of a DNase step before cDNA formation
 - b. Careful PCR primer design
 - c. Adjustments to PCR amplification conditions
4. Beta-actin was the most appropriate housekeeping gene

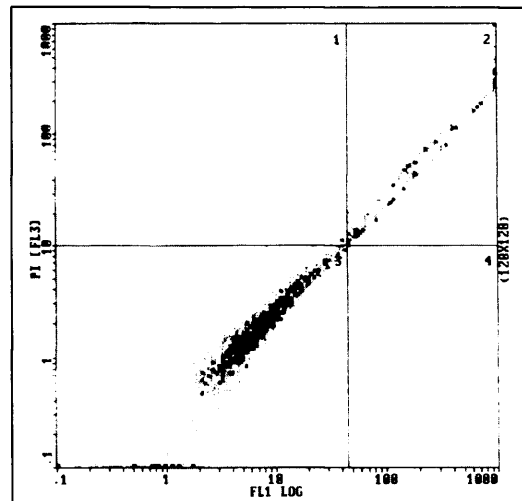
3.6 Development of islet apoptosis assay

Apoptosis is an important endpoint for the evaluation of islet “well being” and previous authors have suggested that increased HIF-1 α expression in islets is associated with increased apoptosis (Moritz, Meier et al. 2002). A number of problems were encountered while assessing apoptosis in dispersed islets by flow cytometry with annexin V (AV) and propidium iodide (PI), a methodology suggested by (Cattan, Berney et al. 2001). The AV/PI technique is designed to detect early apoptosis in cells and should differentiate between healthy, apoptotic and non viable/late apoptotic cells. The assay relies on the binding of a fluorochrome tagged AV to phosphatidyl serine residues on the cell surface. Phosphatidyl serine is a phospholipid which is translocated from the inner part of the cell membrane to the outer surface in early apoptosis and identifies the apoptotic cell for phagocytosis by macrophages (Fadok, Voelker et al. 1992). Propidium iodide is a cell exclusion dye and is unable to enter viable cells. In non-viable cells, propidium iodide enters the cell, binds to DNA and the complex emits light in the red/orange spectrum when excited by blue light (488nm). Therefore, the assay should produce three populations of cells:

Cell population	Signal detected	
	AV	PI
Healthy viable cells	<i>dim</i>	<i>dim</i>
Early apoptotic cells	<i>bright</i>	<i>dim</i>
Late apoptotic/non viable	<i>bright</i>	<i>bright</i>

When islets were assayed using this methodology, the unstained control cells produced a good baseline signal but negative and positive control samples did not produce good bright signals for either annexin V (FITC – conjugated) or propidium iodide positive cells. An increase in voltages did not augment the disparity between bright and dim signals and the histogram of sensors produced a picture of signal overlap (Figure 3.14), despite a high level of compensation (~30-40%) and choosing sensors with disparate wavelengths.

Figure 3.14: A histogram plot of FL1 (annexin V-FITC) against FL3 (propidium iodide) when analysing dual stained cells. The histogram shows overlap of signal between sensors therefore producing a linear pattern on the histogram.



Repeat experiments produced similar histograms, regardless of alterations in sensor voltages and compensation. A check of the flow cytometer proved that all the band pass and dichroic long pass filters were correctly positioned, so the machine was checked with a known positive control for FL1. Due to the small number of cells obtained from dispersed rat islets and the possibility of interference from poorly dispersed cells (i.e. more than one cell passing the laser at a time), peripheral blood mononuclear cells were chosen as experimental cells and CD4-FITC was chosen as a reliable positive control for the FL1 sensor. Peripheral blood mononuclear cells were also used to reassess the AV/PI kit, with positive control cells induced by incubation with hydrogen peroxide (Figure 3.15). In addition, the cell exclusion dye was changed to 7AAD, a marker with a significantly higher wave-length of emission spectra than FITC, which can be detected by the FL4 sensor and allow a greater disparity between the two positive emission spectra.

The 7AAD cell exclusion dye (Beckman Coulter, UK) produced a bright signal and, when compared to the PI from the Roche Diagnostics kit and fresh PI from our own laboratory, it was found that the Roche Diagnostics PI was the only chemical not to provide a bright signal. We concluded that the problem with the PI signal was probably due to the concentration or integrity of the PI supplied by Roche Diagnostics and therefore, further experiments were carried out with freshly made PI.

Figure 3.15: Flow cytometry histograms from experiments with peripheral blood mononuclear cells. Each panel consists of 4 histograms which are, from left to right; forward scatter versus side scatter, signal count versus signal intensity on FL1 sensor (FITC), signal count versus signal intensity on FL2 sensor (PI) and signal intensity on FL1 sensor versus signal intensity on FL2 sensor. Row a: histograms for unstained cells. Row b: histograms for CD4 – FITC stained cells, proving the FL1 sensor works with limited overlap into FL2. Row c: histograms for hydrogen peroxide treated peripheral blood nononuclear cells stained with AV-FITC and PI, proving the apoptosis assessment can work on cells which do not require prior dispersal.

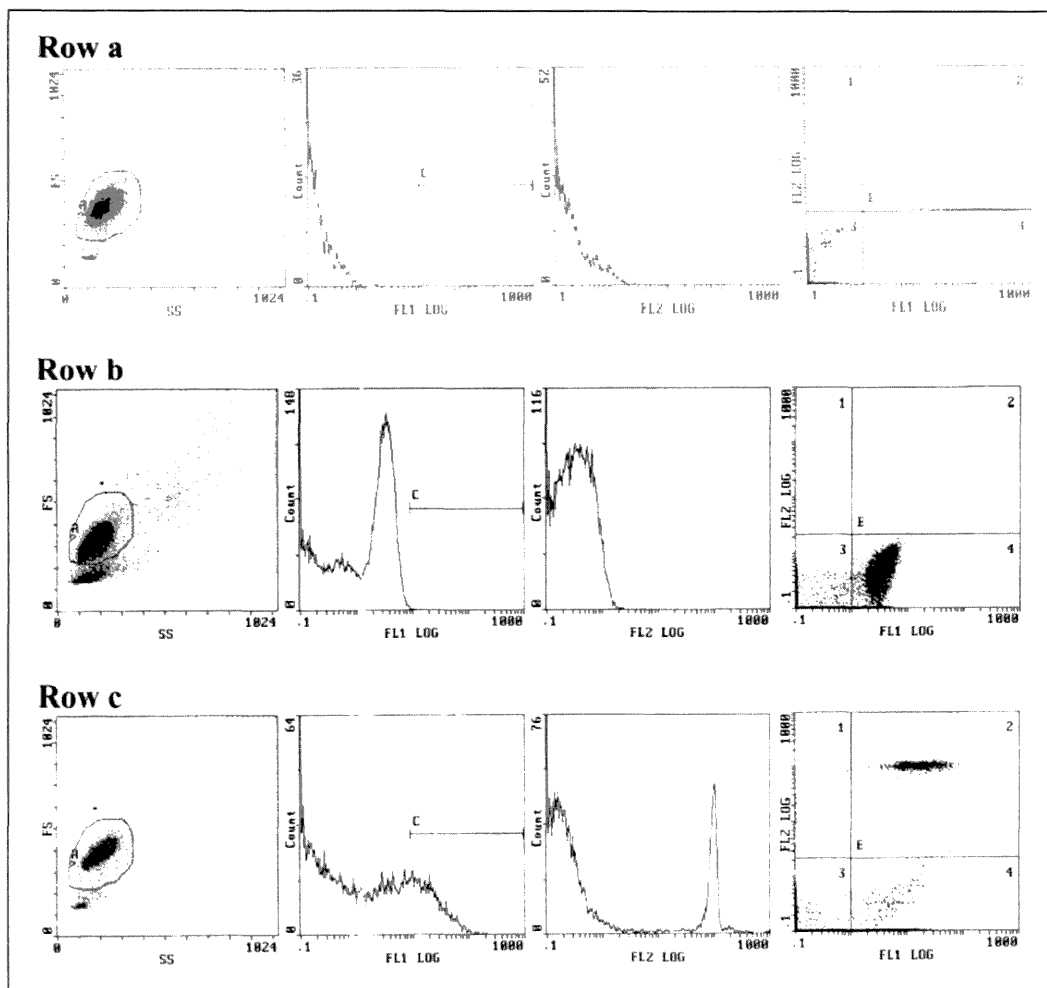
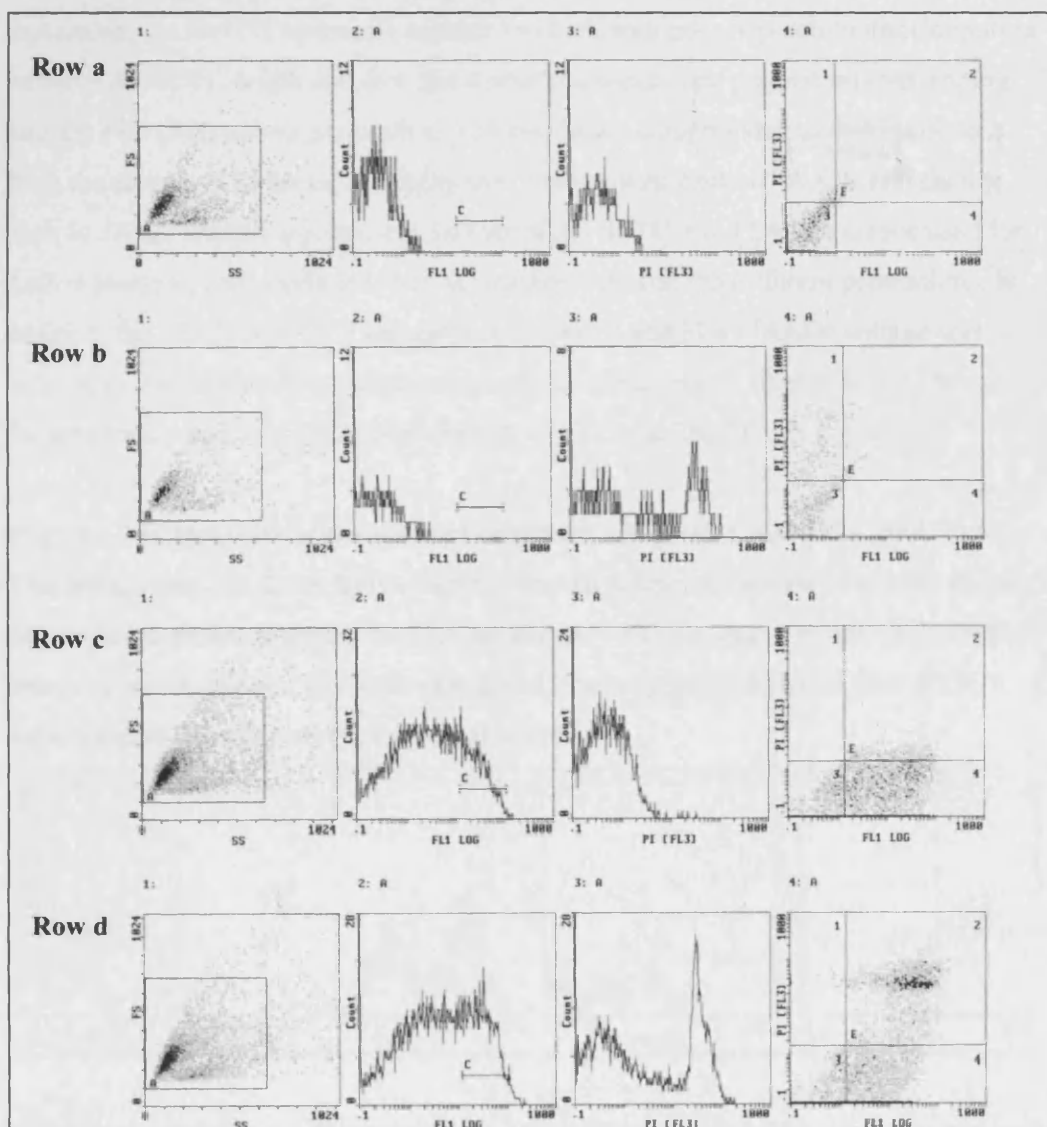
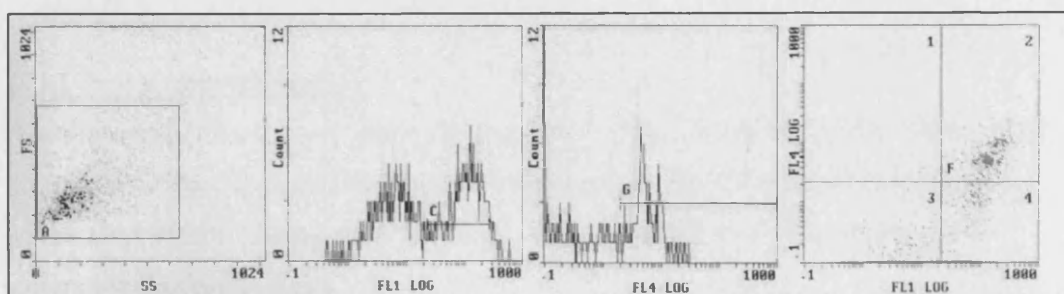


Figure 3.16: Flow cytometry histograms from experiments with dispersed islets. Each panel consists of 4 histograms which are, from left to right; forward scatter versus side scatter, signal count versus signal intensity on FL1 sensor (AV-FITC), signal count versus signal intensity on FL3 sensor (PI) and signal intensity on FL1 sensor (AV-FITC) versus signal intensity on FL3 sensor. Row a: unstained cells, b : PI stained only, c: AV-FITC stained only and d: dual stained.



With the success of running the apoptosis protocol on peripheral blood mononuclear cells, dispersed islets were re-assayed with AV-FITC and fresh PI (Figure 3.16). Unfortunately, the AV-FITC signal did not show any improved disparity between bright and dim signals, although there was some positive signal shift in stained cells. This was further compounded by some overlap of the FITC signal into FL2 and FL3 that proved difficult to compensate for. Although the dual stained plots (Row d, Figure 3.16) appear reasonable, the level of apoptosis appears too high with poor population discrimination between AV-FITC bright and dim and a poorly compensated population overlapping into the FL3 (PI) positive population. The two issues compounded to distort the data from the assay and so the cell viability dye was changed back to 7AAD. The change back to 7AAD limited any spectral overlap of AV-FITC into FL4 (the sensor used for 7AAD analysis) and provided better delineation between the different populations. In addition, the FL1 (AV-FITC) voltage was increased and FL4 (7AAD) voltage was reduced to a minimum, in order to reduce any spectral overlap (figure 3.17). Therefore, the apoptosis assay was performed using AV-FITC and 7AAD.

Figure 3.17: Apoptosis assessment of dispersed islets using AV-FITC and 7AAD. The histograms are, from left to right; forward scatter versus side scatter, signal count versus signal intensity on FL1 sensor (AV-FITC), signal count versus signal intensity on FL4 sensor (7AAD) and signal intensity on FL1 sensor (AV-FITC) versus signal intensity on FL4 (7AAD) sensor.



3.7 Assessment of islet viability

The AV/PI method for the assessment of apoptosis in islets was not thought to provide an accurate representation of islet well being, due to apoptosis induction during islet disruption and the issues of signal discrimination. Therefore, islet viability was investigated as an endpoint to study the effects of desferrioxamine treatment on the integrity of islets.

The main limitation to assessing viability was the availability of large numbers of islets. Although measurement of viability in human preparations is assessed by the percentage of viable islets, this technique could not be translated to the experimental situation as large numbers of islets would be required to gain sufficient sample. Because each rat isolation only provides 400-500 islets, a large number of rats would be required purely for viability assessment, leaving few islets for experiments. The flow cytometry method using disrupted islets and PI can be performed on small numbers of islets but the disruption of islets was thought to reduce cell viability, as outlined above. The assessment of whole islets obviates the disruption issue but requires imaging of cells within the three dimensional structure of islets. The standard cell exclusion dye, trypan blue, could not be used for these experiments as interpretation of staining within a three dimensional structure by plain light microscopy techniques would be almost impossible. Therefore, two colour fluorescent viability of whole islets was employed and compared to the flow cytometry technique.

3.7.1 Flow cytometry method

The dispersed islets stained well with propidium iodide, producing similar traces to the FL3 graph in the above section. There was good distinction between PI bright (non viable) and PI dim (viable) signals and, as only one colour was being observed, no compensation was required.

3.7.2 Intact islets

Initial experiments with fluorescein diacetate and acridine orange were undertaken to establish a quantitative measurement for the viability of cells within a small population of islets. These experiments were undertaken using a similar methodology as described in chapter two but two different viability dyes were used at different concentrations, 5 μ l fluorescein diacetate (1 μ g/ml in acetone) or 1 μ l of acridine orange (either 2mg/ml, 0.2mg/ml or 0.02mg/ml). The slides were counterstained with 2.5 μ l of propidium iodide (250 μ g/ml).

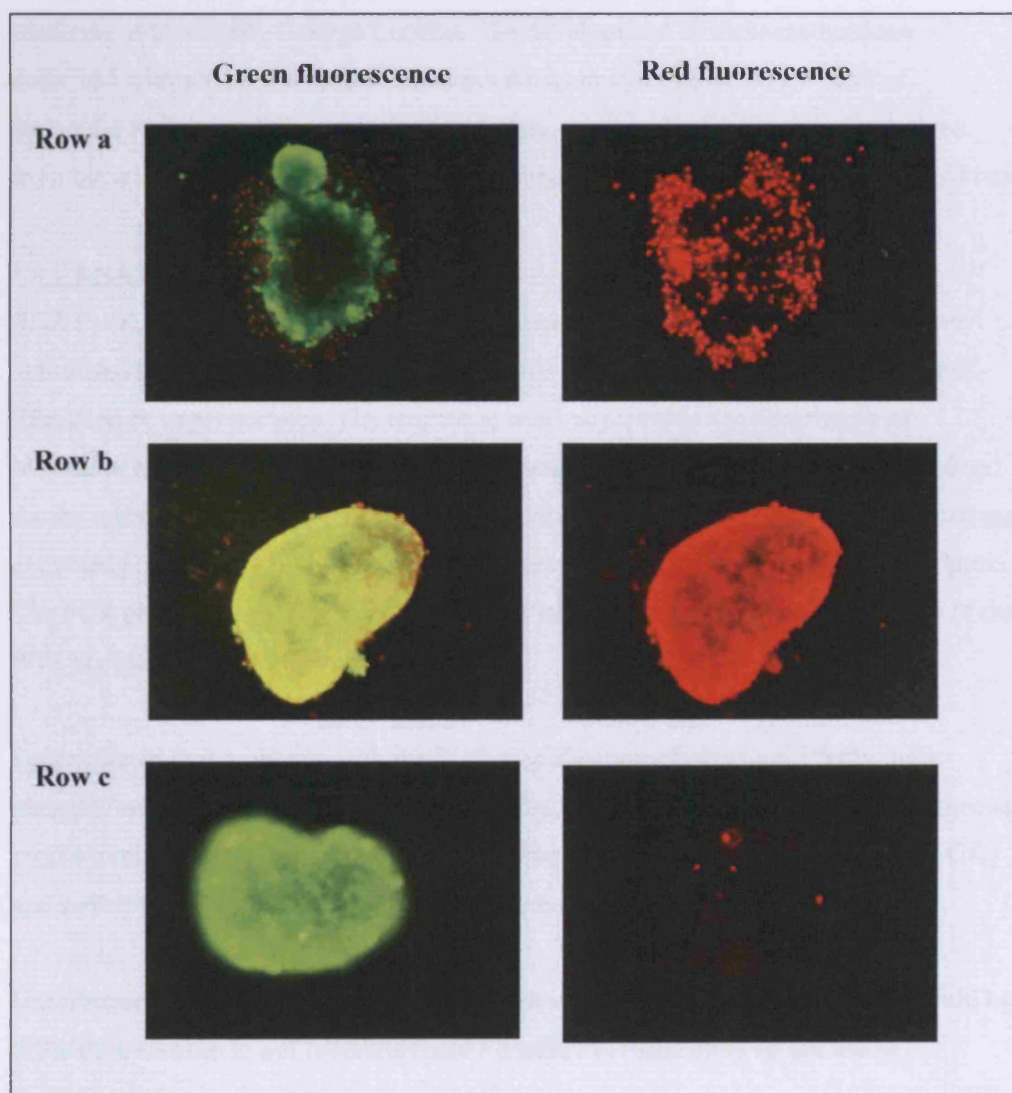
The fluorescein diacetate staining showed poor penetration into the core of the islets and did not provide visualisation of individual cells within the islet, therefore making assessment of the percentage of viable cells impossible (figure 3.18, Row a). Acridine orange (AO) penetrated into the islets better than fluorescein diacetate and highlighted the viable nuclei well but higher concentrations (2mg/ml and 0.2mg/ml) were associated with an increase in background fluorescence (figure 3.18, Row b). This higher level of background orange/red fluorescence interfered with the PI assessment of viability and the increased orange/green fluorescence hindered the counting of viable cells. The background fluorescence could not be reduced by washing of the cells prior to analysis and therefore lower doses of acridine orange were used for experiments. The lower concentrations of AO did not interfere with PI assessment of viability but nuclei of live cells were not highlighted in green, although viable cells could still be counted by green cytoplasmic fluorescence (figure 3.18, Row c). This allowed the imaging and counting of live cells within a focal plane.

3.7.3 Conclusions from assessment of islet viability

These experiments showed that:

1. Islet viability could be reliably assessed using flow cytometry with PI.
2. Islet viability in whole islets could be assessed with low concentration AO and PI, by counting the number of viable cells within a focal plane.

Figure 3.18: Assessment of viability in whole islets stained with; Row a: Fluorescein diacetate and propidium iodide (H_2O_2 treated), Row b: High dose acridine orange (~200ng/ml) and propidium iodide (control fresh) and Row c: Low dose acridine orange (~2ng/ml) and propidium iodide (control fresh).



3.8 Development of in-situ techniques

The exploration of endothelial origin and the site of growth factor expression required the use of in-situ techniques. The in-situ techniques were not established within our laboratory and were learned at Cancer Research UK and the department of Molecular Medicine at University College London. The development of these methods are described within their own section because the techniques relied on a hybrid of molecular biology and histology. Unfortunately, no meaningful results were derived from these methods but a brief description of the development process will be listed here.

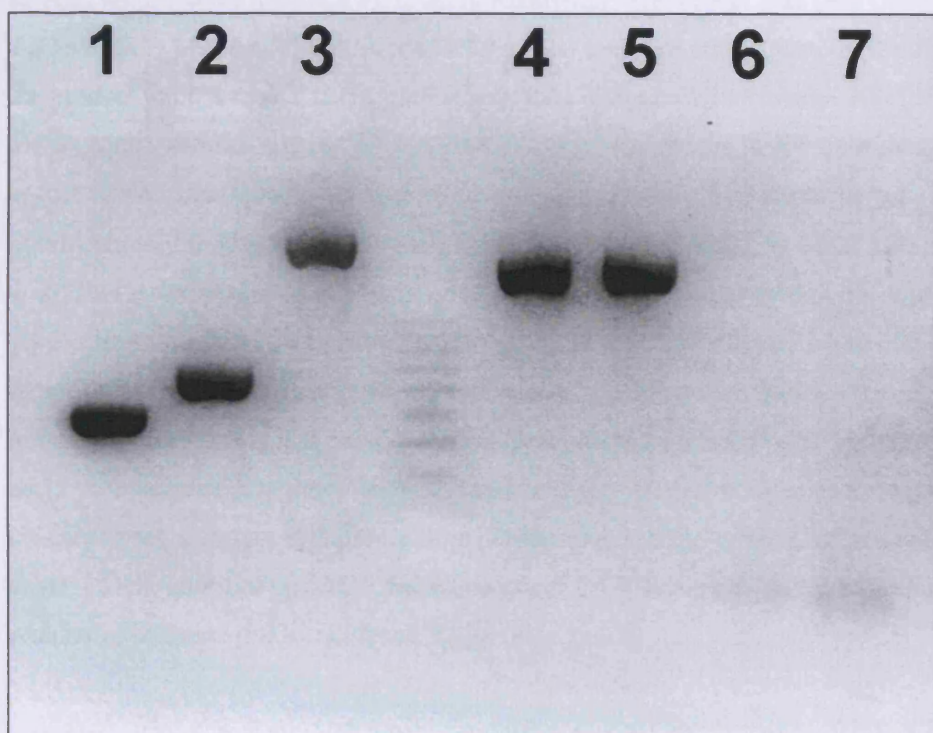
3.8.1 RNA in-situ hybridisation

RNA in-situ hybridisation requires the formation of cDNA labeled probes which are hybridised to mRNA within tissue sections, in order to spatially describe the point of formation of target proteins. The technique was learnt within the department of Molecular Medicine at University College London and IMAGE clones were purchased for the relevant mRNA sequences. The IMAGE clones were initially broth amplified and extracted plasmid was further amplified by standard PCR techniques with M13 primers. The PCR products were sequenced and found to be of the correct sequence. Some of the PCR products are illustrated in figure 3.19.

I encountered two problems with the PCR amplification of plasmids. Firstly, two plasmids were empty vectors, although the galactosidase gene was disrupted. The second problem related to GC rich sequences which required the addition of Q (Quiagen, UK) and switch to Quiagen PCR amplification products.

Unfortunately, after collecting the sequences it was decided that the technique would be difficult to transfer to our laboratory and I decided to focus more on the use of desferrioxamine.

Figure 3.19: Gel electrophoresis of purified IMAGE clone sequences. Lane 1:Ang 2, lane 2:bFGF, lane 3:Ang 1, lane 4:TGF β , lane 5: VEGF, lane 6 and 7 are empty vectors from sequences supplied as human and rat insulin respectively.

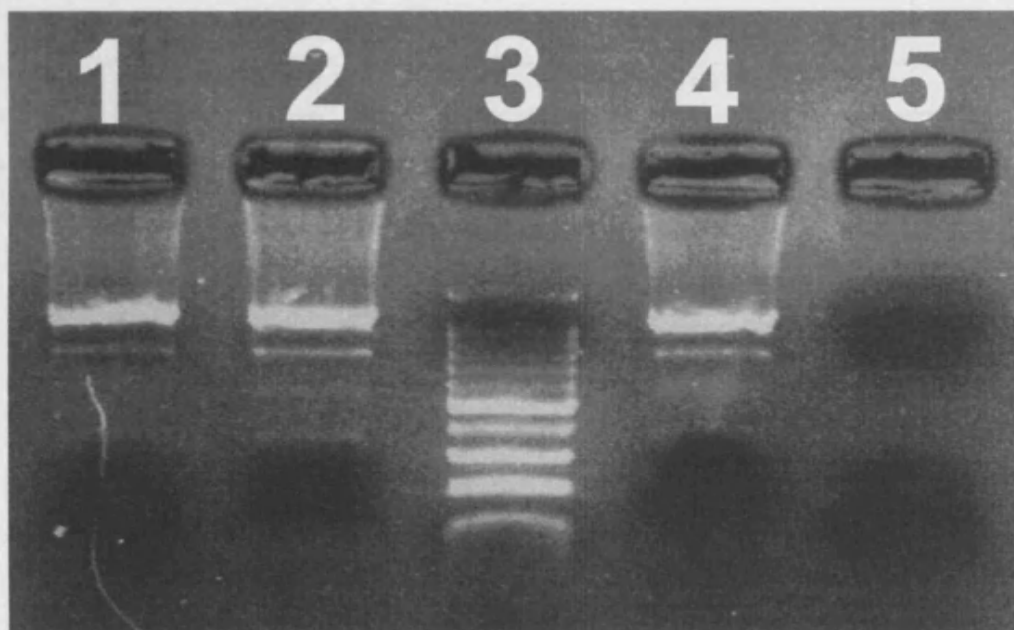


3.8.2 Fluorescent in-situ hybridisation of the Y chromosome

A method was required for tracking the origin of transplanted islet endothelium. I considered the use of xenogenic models, green fluorescent protein animals and reporter genes, such as LacZ, but there was limited experience or scope to transfer these techniques to our laboratory. I therefore tracked the use of Y chromosome FISH to a group within cancer research UK and visited the lab to learn the technique. Their initial results with mouse Y chromosome FISH was good and after some optimisation, they managed to use the rat Y chromosome sequence to set up the technique.

The rat Y chromosome sequence was sourced from Dr Barbara Hoebe in the Netherlands and was supplied in a pUC13 plasmid. Initial attempts to insert this vector into E Coli and amplify the sequence by broth failed. Therefore, the sequence was directly amplified by standard PCR using Reddymix PCR master mix (AB Gene, Epsom, UK) and M13 primers. The PCR product was just over 1kb and sequencing confirmed the product to be the rat Y chromosome sequence with accession number X80155.1. As the sequence was 1kb and probes of 200-500bp are required for in-situ hybridisation, the sequence was digested with DNase to develop short segments of the probe for hybridisation. Initial experiments were performed by incubating 1µg of the 1kb probe with DNase (Invitrogen, UK) for varying periods of time and analysing the digests by gel electrophoresis. The incubation times of 7.5, 15 and 22.5 minutes led to complete digestion of the 1kb probe and subsequent incubation times were reduced to 1, 2 and 5 minutes. Unfortunately, the 1 and 2 minute times did not produce sufficient digestion and 5 minutes digestion times caused complete digestion of the Y sequence (figure 3.20). Despite trying a variety of different time points, a reproducible digestion could not be achieved and I noticed that M13 amplification of the Y sequence had now produced a number of different products (figure 3.20).

Figure 3.20: DNase digestion of rat Y sequence. Lane 1 is the 1kb sequence amplified by M13 primers. Lane 2,4 and 5 are the same sequence digested with DNase for 1,2 and 5 minutes respectively. Lane 3 is a 100bp ladder.



The DNase digestion did not allow the formation of small fragments for staining and therefore internal primers were designed using Primer Express (Applied Biosystems) software and the sequence of the Y probe. Five sets of internal primers were created with products of 200-500bp which were distributed at different points within the Y probe. The primers and products are outlined in table 3.8.

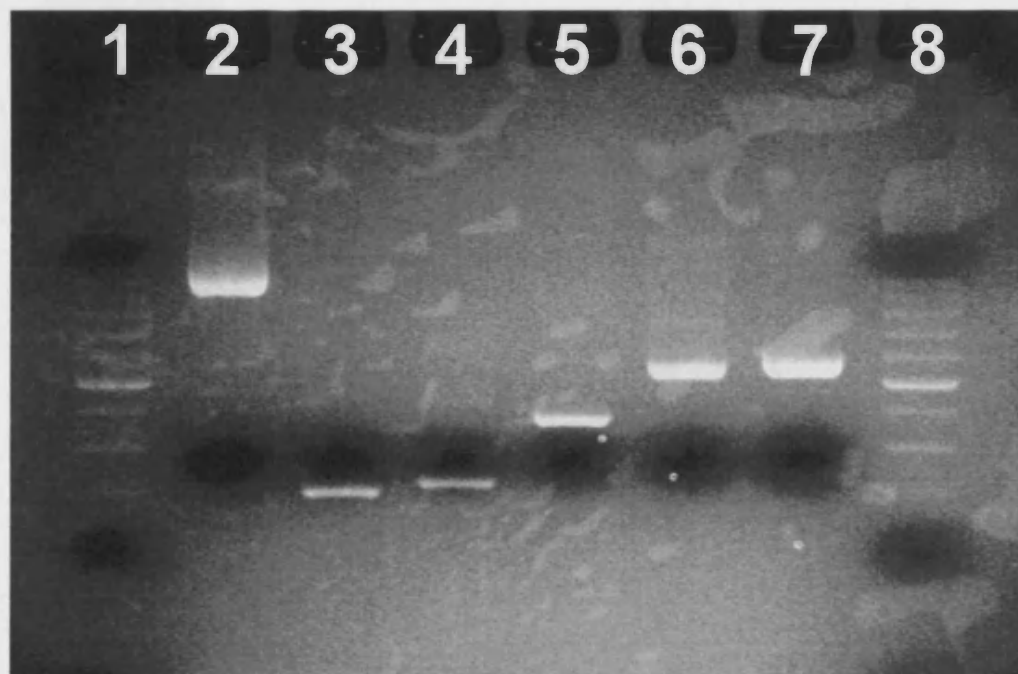
Table 3.8: Internal primers of the Y chromosome and their product length.

Primer number	Forward primer	Reverse primer	Product length
1	CCCCTTTTTCCTTTTACAAA	TCTCTTCAGCTTCAAACCTCAAGT	204bp
2	TCGTTCCAAGCGGCA	GCTCTTCCCCCTTGGAGA	215bp
3	CTTGAGTTTGAAGCTGAAGAGAA	TGCCGCTTGGAACGA	377bp
4	GCCCCTTTTTTCCTTTTACA	TGCCGCTTGGAACGA	555bp
5	CTTGAGTTTGAAGCTGAAGAGAA	GCTCTTCCCCCTTGGAGA	577bp

In view of the multiple products from the amplified Y probe, sequences were amplified directly from the plasmid. All of these primers produced the correct product lengths when the y probe was amplified by standard PCR (figure 3.21).

Figure 3.21: Y probe amplification using different primers and standard PCR.

Lane 2: M13 primer, Lane 3: primer 1, Lane 4: primer 2, Lane 5: primer 3, Lane 6: primer 4, Lane 7: primer 5. Lanes 1 and 8 are 100bp ladder.

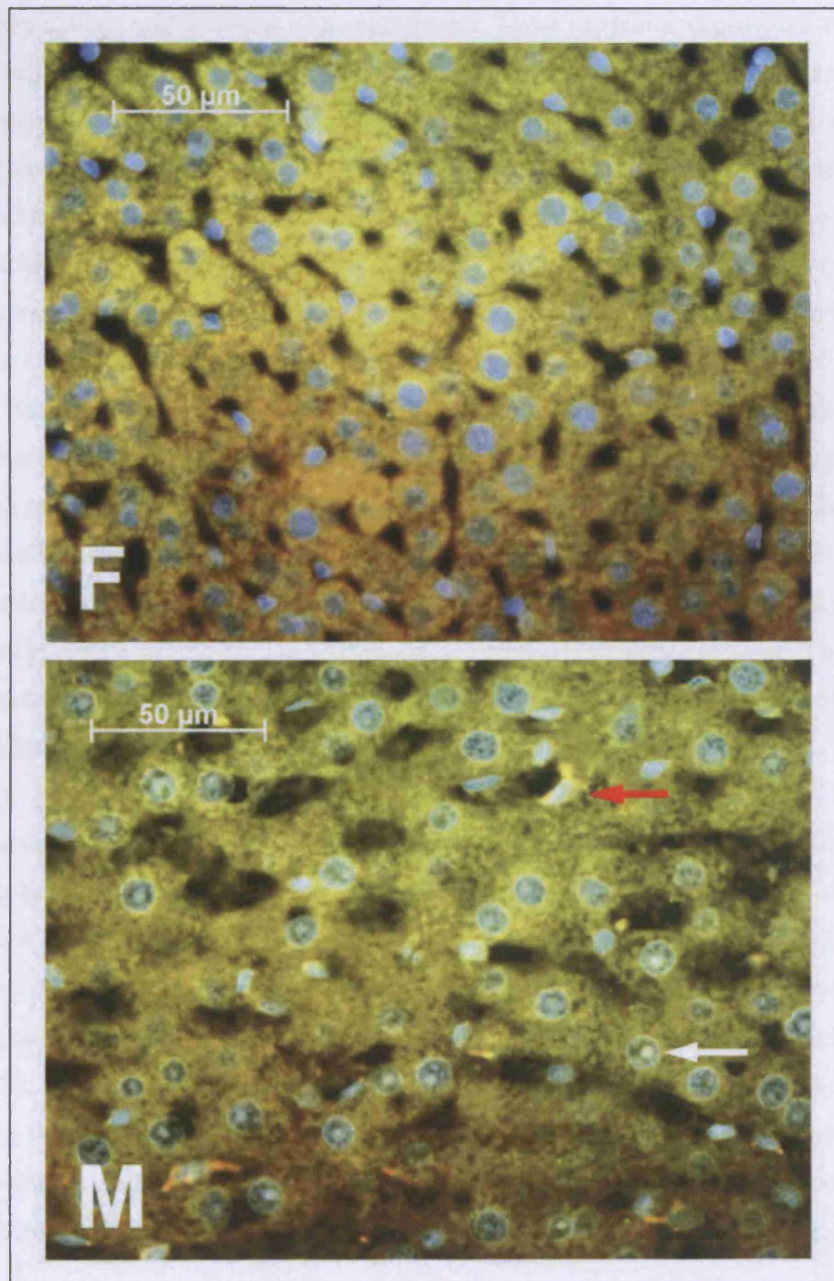


These primers were subsequently used for the formation of digoxigenin labeled sequences and anti-dig Fab fragments were used as a second layer, in order to amplify the signal. The first experiments were designed to optimize digestion time with pepsin and time points of 1,2,5,10 and 15 minutes were used. The 15 minute time point caused over digestion of tissue and loss of cellular architecture. The lower time points did not have obvious staining within nuclei. Further experiments were carried out using different pepsin digestion times, different probes, increasing concentration of probes and Fab fragments. The 12 minute digestion time with the 577bp probe from primer set 5 gave the best signal but the signal remained weak and did not obviously stain fusiform nuclei of endothelium (figure 3.22). In addition, not all cells stained with the Y probe which probably reflects levels of tissue sectioning, where the part of the nucleus containing the Y chromosome may not have been on the tissue section.

3.8.3 Conclusions from in-situ hybridisation techniques

1. The Y chromosome probe could be amplified from plasmid and was of correct sequence.
2. Small probe lengths were best formed by internal primer sets
3. Signal amplification was poor and further optimization is required before using as a robust technique.
4. The sectioning levels of tissue may reduce accuracy of male cell identification.

Figure 3.22: Y chromosome FISH of female (F) and male (M) hepatic tissue. The male nuclei are stained with small dots within the centre of the nuclei (white arrow). The staining was not 100% consistent and the compact fusiform nuclei of endothelium was not obviously stained (red arrow).



3.9 Discussion

3.9.1 Islet transplant model

In-vivo models are the mainstay of islet transplant research and are necessary for proof of principle when manipulating islet engraftment. These experiments provided evidence for the reproducibility of our transplant model and the additional step of transplanting islets only into the right branch of the portal vein. The right branch technique was further extended to allow sex mismatching and good resolution of blood glucose post transplantation was achieved in female recipients. These results suggest that the sex mismatching does not affect the clinical outcome after transplantation and there is little evidence of sensitisation to the minor H-Y antigen. The initial difficulties experienced with the technique were probably related to the small size and frailty of female recipients, caused by high doses of streptozotocin and halothane anaesthesia. The 55mg/Kg dose of streptozotocin, used for inducing diabetes in male recipients, proved too high and resulted in some female recipients dying from severe diabetes. This complication is likely to be due to the high fat to lean body mass ratio of female animals (as streptozotocin is lipophilic) and the dose reduction resulted in a safer induction of diabetes in female recipients. These experiments provided evidence to suggest that our optimised model was a robust and reproducible technique for the examination of islet transplantation.

The resolution of blood glucose is an important endpoint for assessing the engraftment of islets but does not examine the mechanisms involved. The extension of our islet transplant model to include histological evaluation provides further endpoints for the analysis of islet engraftment. The poor delineation of islet endothelium has previously limited such studies but was resolved by using a specific marker (Mattsson, Carlsson et al. 2002). When endothelial delineation was combined with simultaneous insulin staining, the composite image provided accurate structural observation of the whole transplanted islet. The initial variability in BS1 staining was reduced by optimization in antigen retrieval and tissue processing, with particular attention to heat treatment. The

subsequent model has provided a robust technique for the assessment of native and transplanted islets.

3.9.2 Growth factor analysis

The hypothesis for desferrioxamine up regulating growth factor expression in islets is that it exerts its effects through the altered expression of hypoxia inducible factor. Hypoxia inducible factor is a heterodimeric transcription factor composed of two basic helix-loop-helix domains (Lando, Gorman et al. 2003). The two domains consist of an alpha subunit, which has three different isoforms, and a beta subunit. The regulation of HIF-1 expression by oxygen is brought about by hydroxylation of proline residues in the oxygen dependent domain of the alpha subunit by HIF α prolyl-hydroxylase (HPH) (Jaakkola, Mole et al. 2001). The hydroxylated alpha subunit is then ubiquitinated by the von Hippel-Lindau protein (Maxwell, Wiesener et al. 1999) and subsequently destroyed by proteosomes. HIF-1 expression is also regulated by the oxygen dependant hydroxylation of asparagine residues by factor inhibiting HIF (FIH-1) within the COOH-terminal transactivation domain (CAD) which acts to inhibit the recruitment of the p300 transactivator necessary for transcription of HIF-1 activated genes (Lando, Peet et al. 2002; Maxwell 2003).

The mechanism of action of altered angiogenic factor expression with desferrioxamine is a reduction in the activity of HPH which leads to limitation of the first step required for eventual destruction of HIF-1 α by proteosomes (Jaakkola, Mole et al. 2001) and inhibition of FIH-1 which limits the inhibition of HIF transcription activation (Lando, Peet et al. 2002; Maxwell 2003). This mechanism is post translational and therefore required a protein or HIF-DNA binding assay to examine the hypothesis in islets. A change in HIF-1 protein concentrations due to DFO treatment would have been an important observation to support the hypothesis for altered growth factor expression within islets. Unfortunately, I was unable to detect HIF-1 expression with the developmental ELISA due to either the quantity of HIF-1 in cell lysates or cross reactivity of the assay. An increase in rodent islet number would not have been practical or ethically justifiable and sufficient human material was not available. An alternative

approach would have been western blot analysis of protein with specific HIF-1 antibodies which may be more sensitive and specific for rat HIF-1. Another cause for the inability to detect HIF-1 with the developmental assay may have been the rapid degradation of HIF-1 during cell lysis. In the presence of oxygen, HIF has a half life of less than 1 minute (Yu, Frid et al. 1998) and it is possible that HIF-1 may have been rapidly metabolised during cell lysis, despite the presence of protease inhibitors and incubation on ice. Therefore, HIF-1 may not have been present in the cell lysates at the time of assay with the developmental kit.

Unfortunately, time and the availability of a suitable assay limited the ability to analyse the expression of HIF-1. Due to the lack of a protein assay, I have relied on the published literature within this area to reinforce the mechanism of DFO up regulating HIF-1 and subsequent down stream growth factors (Wang and Semenza 1993; Wang, Jiang et al. 1995; Cooper, Lynagh et al. 1996; Gleadle and Ratcliffe 1997; Linden, Katschinski et al. 2003).

The analysis of VEGF protein in culture supernatants did not require additional optimisation and supernatant dilution was not required before analysis by ELISA and no VEGF expression in the culture medium. Unfortunately, the protein analysis of other growth factors was not as simple and the bFGF estimations required considerable optimisation before analysis could be performed. The HGF ELISA did not detect any HGF in the culture supernatant and was probably due to poor cross reactivity of the human HGF ELISA or the low levels of HGF in culture supernatants.

3.9.3 Islet integrity analysis

Islet viability is integral to assessing islet well being before transplantation and is usually measured in a population of islets by either calorimetric (Kumar, Delfino et al. 1994) or fluorescent visualisation (Bank 1987; Miyamoto, Morimoto et al. 2000; Barnett, McGhee-Wilson et al. 2004). These methods either rely on a cell exclusion dye alone (trypan blue), a chemical which is diffusible and undergoes a change within viable cells to produce a colour (MTT test) or a combination of the two (fluorescein

diacetate/propidium iodide or acridine orange/propidium iodide). The tests allow rapid assessment of viability within a population of islets (Bank 1988) but require large numbers, at least 200, to accurately sample the population. The number of islets required for viability assessment precludes the use of this technique for rodent models and therefore, a new method was necessary for assessing the viability of cells within islets. These experiments demonstrated that islet viability could be assessed in a small population of islets and the measurement of cellular viability within islets could be measured both manually in intact islets and automatically in dispersed islets.

Islet viability provides a gross assessment of cellular well being but more subtle processes leading to islet death may not be obvious whilst performing such crude analysis. Apoptosis assessment provides a more subtle analysis of islet wellbeing and is able to quantify less obvious insults that lead to programmed cell death. Unfortunately, apoptosis assays are hard to perform on islets because they are not single cells but a conglomeration of cells. Some authors have suggested using flow cytometry with annexin V and propidium iodide on disrupted islets (Cattan, Berney et al. 2001) but this method reports a high rate of apoptosis (~20%), which does not fit with the rate of loss of islets in culture or the percentage of apoptotic nuclei (small bright nuclei on PI staining) visualised in whole islets. Our experience with this technique would suggest that a high level of annexin V binding to disrupted islets is probably due to exposure of phosphatidyl serine residues on the membranes of islet cells during the disruption process. This would explain why disrupted islets produce a non-specific increase in annexin expression with no clear population of bright or dim annexin stained cells, which contrasts with the two clear populations generated by analysis of peripheral blood mononuclear cells. In addition, the disruption of cellular adhesion molecules has been shown to induce apoptosis in adherent cells (Erez, Zamir et al. 2004) and in islets, a study using confocal microscopy suggested that disruption non significantly increased apoptosis from 11.5% to 17.9% (Boffa, Waka et al. 2005). Therefore, the disruption process may induce programmed cell death and probably overestimates the level of apoptosis within islets.

The annexin V/PI method for assessing islet apoptosis is fraught with difficulties and probably overestimates the rate of apoptosis within islets. A technique using TUNEL or caspase analysis may represent a better technique.

3.9.4 In-situ techniques

In situ hybridization is a difficult technique to master and further optimization is required before the Y chromosome technique can be used to track endothelial origin. The signal within the nuclei was weak but the observation of a signal meant that the probe was entering the nuclei and further optimization of hybridisation was not required. Therefore, the signal needed to be amplified and may have been achieved by using a different colour or fluorophor, as red may have been of significantly disparate wavelength and shown up better on the blue nuclear background. Secondly, the entire 1kb sequence was not used as a probe and if this size of probe could be hybridised into the cell, this option may have increased the signal intensity. Nick translation of the probe with random cutting could also have painted the Y chromosome better than specific short sequences and may have provided a better method for greater chromosome localisation. After achieving greater chromosome hybridization, signal amplification could also be achieved by either using a higher power of fluorochrome excitation, such as confocal microscopy, or by using immunohistochemistry instead of using fluorescence. Even with optimal visualization, the issue of slicing through nuclei may make the technique less reliable and a large number of sections would need to be imaged. If this was the case, a cytosolic marker such as GFP may be a better way to reliably delineate endothelial origin.

3.9.5 Conclusions

These experiments optimized the techniques necessary for the investigation of islet transplantation and were used as the basis of the experiments outlined in the following chapters.

Chapter 4 – Results:

The revascularisation of intraportally transplanted islets

4.1 Introduction

The lack of functional vasculature within islets during the immediate post transplant period is the most likely cause for the large loss of islet mass post transplantation (Menger, Yamauchi et al. 2001). The revascularisation of transplanted islets is probably key to initial β -cell mass survival and a better understanding of the structural changes during this period is required in order to improve islet survival.

The original description of transplanted islet revascularisation was published in 1977 using a rat model of intraportal transplantation (Griffith, Scharp et al. 1977). The morphology of the islets was described by using a series of different stains for the various cellular components within islets and compared to haematoxylin and eosin and electron microscopy images, in order to identify vascular structures. Transplanted islet revascularisation has subsequently been described using dorsal skin fold models with direct in-vivo visualisation of the graft (Vajkoczy, Menger et al. 1995; Beger, Cirulli et al. 1998) and in kidney subcapsular models with retrieval of the graft and immunohistochemistry staining with Bandeiraea Simplicifolia (Carlsson and Mattsson 1993; Carlsson, Palm et al. 2002; Mattsson, Jansson et al. 2003). Both these techniques have their advantages but do not allow simultaneous visualisation of both endothelium and endocrine mass. The dual Bandeiraea Simplicifolia and insulin fluorescent staining enables simultaneous imaging of both components allowing accurate analysis of islet structure and estimation of vessels per endocrine area. This technique has been further enhanced to estimate the percentage vascular area of islets (as defined by the ratio of endothelial to endocrine area), calculate vascular densities and branching index. This novel staining technique combined with image processing has now facilitated the quantitative analysis of islet revascularization and should enable direct numerical comparison of methods used to alter revascularization.

The application of these new staining and analysis techniques to pancreatic tissue has allowed the visualization and quantification of islet vasculature within the native pancreas. Native pancreatic islet vascular architecture is complex (Bonner-Weir and Orci 1982) and analysis of islets within the pancreas facilitates direct quantitative comparison with islets post transplantation. The changes in islet vasculature after transplantation have been examined using a variety of models and have suggested that islets revascularise poorly with decreased oxygen tensions and capillary density when compared to native islets (Carlsson and Mattsson 1993; Carlsson, Palm et al. 2000; Carlsson, Palm et al. 2001; Carlsson, Palm et al. 2002; Jansson and Carlsson 2002; Mattsson, Jansson et al. 2002). Although one study did look at the change in vascular density after intra-portal transplantation (Mattsson, Jansson et al. 2002), most of the studies have been performed in kidney subcapsular models. However, kidney subcapsular and dorsal skin fold transplantation of islets may not provide an accurate model for the assessment of islet revascularization. Therefore, the selective intra-portal transplant model and dual insulin/endothelial staining technique was applied to analyze changes in islet vasculature post transplantation. This chapter describes the application of this model to analyze post transplant changes in vascular architecture of intra-portal transplanted islets.

4.2 Change in clinical parameters post sex-mismatched right branch transplantation

Diabetes was induced with STZ in 30 female Lewis rats. The animals had a mean body weight $190.6 (\pm 1.9)$ g at the time of streptozotocin injection which fell to $174.4 (\pm 1.3)$ g with a mean blood glucose of $26.2 (\pm 0.8)$ mmol/l, at the time of transplantation.

Islets were isolated from 60 male Lewis rats (~250-280g) and transplanted into the right portal branch of the female Lewis rats. The females received a mean dose of $752 (+/- 33.3)$ freshly isolated, hand picked islets. Four recipients were sacrificed while under anaesthetic fifteen minutes after transplantation and the remaining recipients were humanely killed at various time points (1, 3, 5, 7, 14 and 30 days) after transplantation. The mean changes in blood glucose and weight for all recipients are displayed graphically in figure 4.1 and 4.2.

Figure 4.1: Change in mean blood sugar over time in all female recipients of male islets via the right branch transplant method. Values are expressed as mean plus standard error.

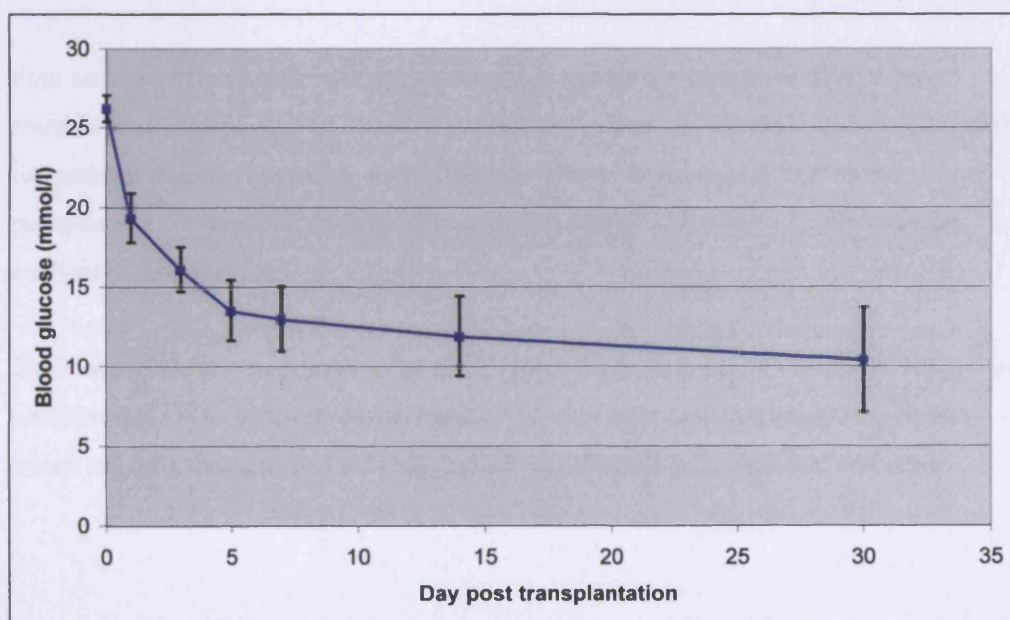
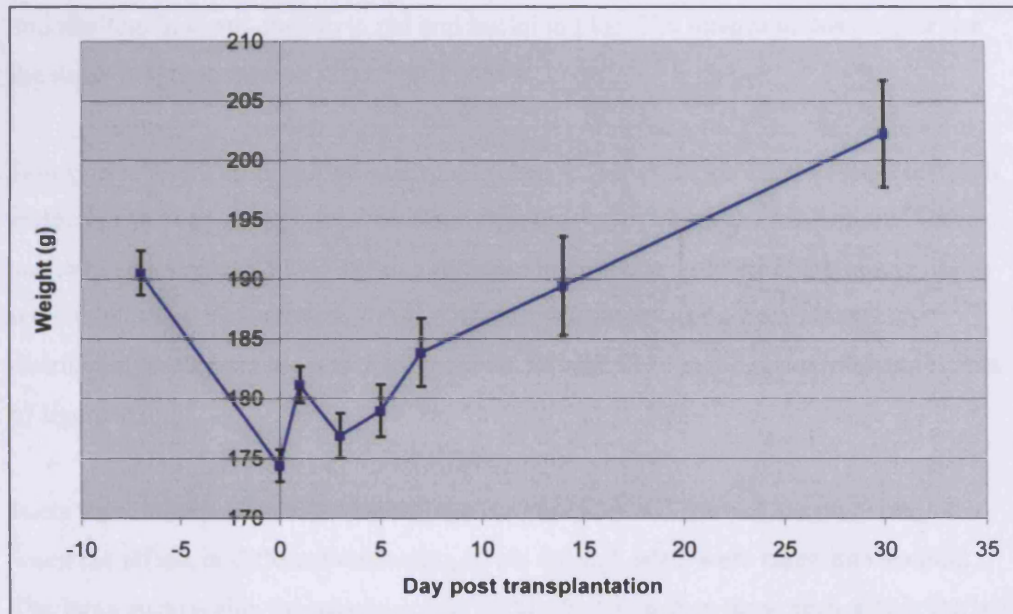


Figure 4.2: Change in mean weight over time in all female recipients of male islets via the right branch transplant method. Values are expressed as mean plus standard error. The sharp rise in weight on the first post operative day probably represents fluid retention and peri-operative intra-peritoneal fluid administration.



Four animals were sacrificed at each time point, except for 14 and 30 days, where 5 animals were sacrificed. The livers were retrieved, processed and analysed histologically for vascular density, branching index, endothelial and insulin area. Four native pancreases were removed from healthy male rats and processed in a similar way for analysis of native islets.

The blood glucose concentrations of transplanted diabetic female recipients corrected in a similar fashion to previous experiments with male right branch transplant recipients which suggests that sex mismatching did not significantly affect clinical outcomes.

4.3 Qualitative histological analysis of native islets

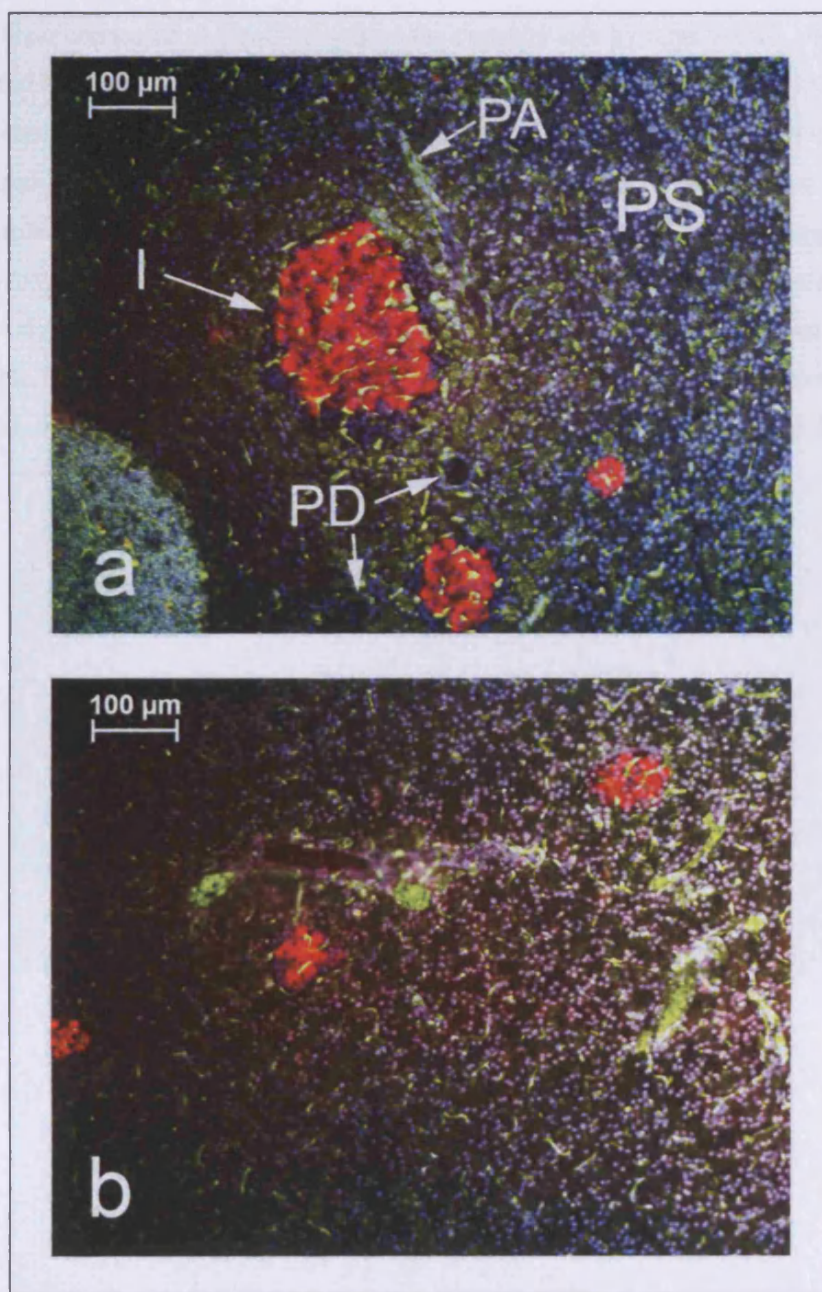
Native pancreases from four healthy male rats were embedded into single blocks and sectioned at 4 μ m. The sections were stained with the dual endothelial and insulin stain and analysed as outlined in materials and methods. This method highlighted endothelium in green, insulin in red and nuclei in blue. The images in this chapter use the same colour scheme to identify structures.

Low power views of the native pancreas (figure 4.3) showed good delineation between endocrine and exocrine tissue with clear visualisation of vascular endothelium. There was weak background FITC staining of exocrine tissue and some FITC staining of the red blood cells within vessels. Areas of dystrophic calcification were also seen distributed throughout the pancreatic stroma, as visualised in the lower left hand corner of figure 4.3a.

Islets were widely distributed throughout the pancreas and were of varying size, even when the effects of different sectioning levels through islets were taken into account. The large islets within the pancreas were generally larger than those seen within the liver post transplant. In addition, the islets within the pancreas had a ring of non-stained (no insulin or background FITC staining) cells around the endocrine core, which is not seen in intra-portally transplanted islets. It is impossible to define these cells without further immunohistology but it is probable that these cells represent the non beta cell mantle of the islet.

The endothelial staining highlighted both large arteries and veins within the pancreas and small capillaries within islets. The most striking vascular observation was that the vessels appeared smaller and less numerous within native islets when compared to islets transplanted to the liver. This was particularly noticeable within the smaller islets, where blood vessels were scarce and barely visible.

Figure 4.3: Low power (x100) view of native islets within a rat pancreas. The islets (I) are stained with insulin in red and endothelium in green. The other visible structures within the pancreatic stroma (PS) are a pancreatic arteriole (PA) and pancreatic ductules (PD).



When islets were visualised at higher magnification, the different vascular networks became clearer. The smaller islets, under 100µm, had a rim of surrounding blood vessels arranged circumferentially around the insulin positive cells. The circumferential blood vessels were connected to blood vessels on the opposite side by large vessels traversing the central beta cell core of the islet. Although there were one or two branches within the central core, there was no obvious glomerular network of vessels- see figure 4.4. The large sized islets (over 250µm) had a different vascular architecture with a less discernable network of circumferential vessels around the beta cell core, although there was evidence of some circumferential vessels outside of the non staining mantle of cells (figure 4.6). In the core of the large islets, there was an obvious glomerular like network of fine blood vessels with few large traversing vessels. The mid or average sized islets (150-200µm) had a mixture of the two different vascular arrangements (figure 4.5).

Figure 4.4: High magnification view (x400) of small (50-100 μ m diameter) native islets within the rat pancreas.

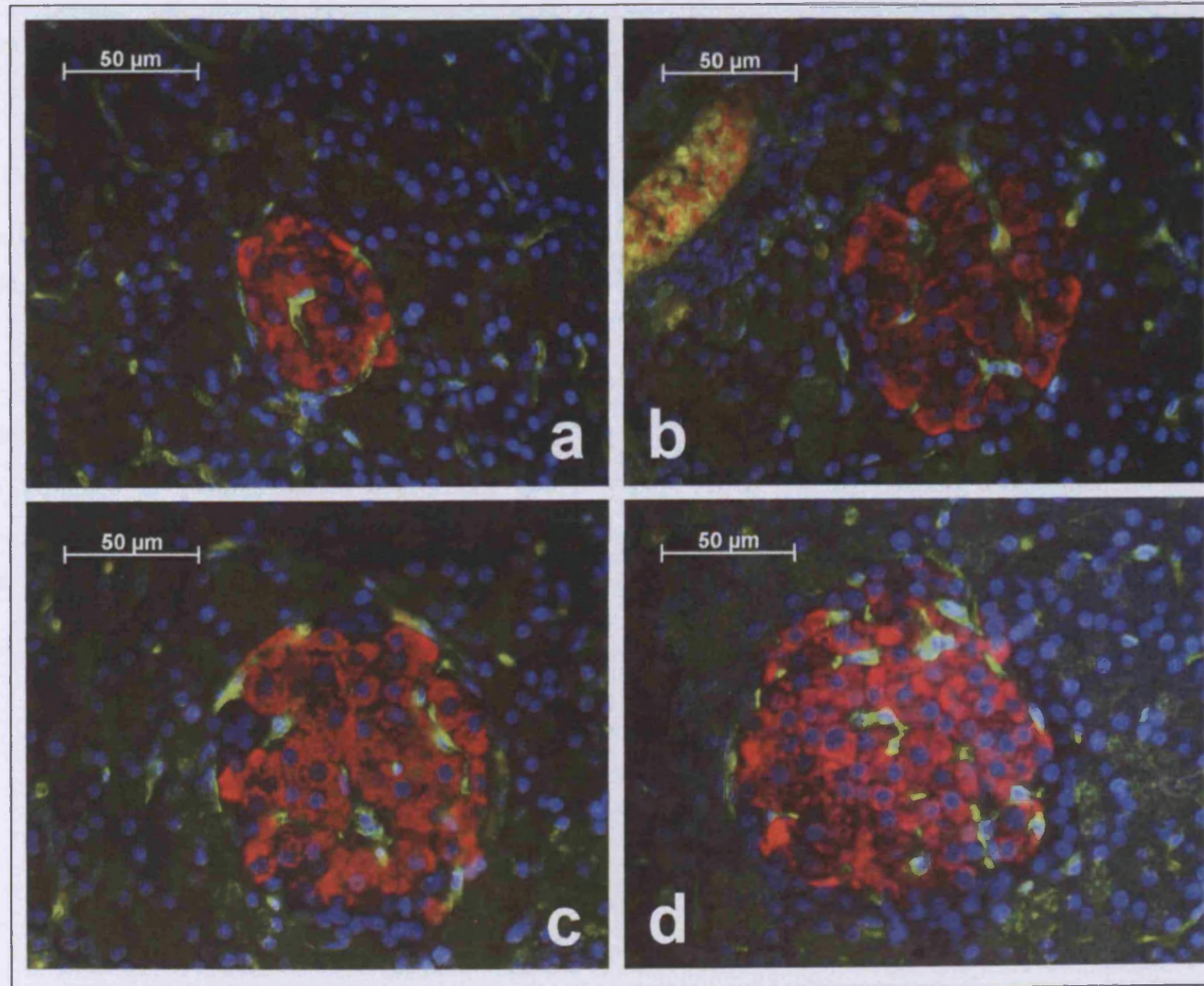


Figure 4.5: Average sized (150-200 μ m) native islets within the rat pancreas.

Image (a) and (b) are the same islet at 200x and 400x magnification respectively. Image (c) and (d) are two different islets at 400x magnification.

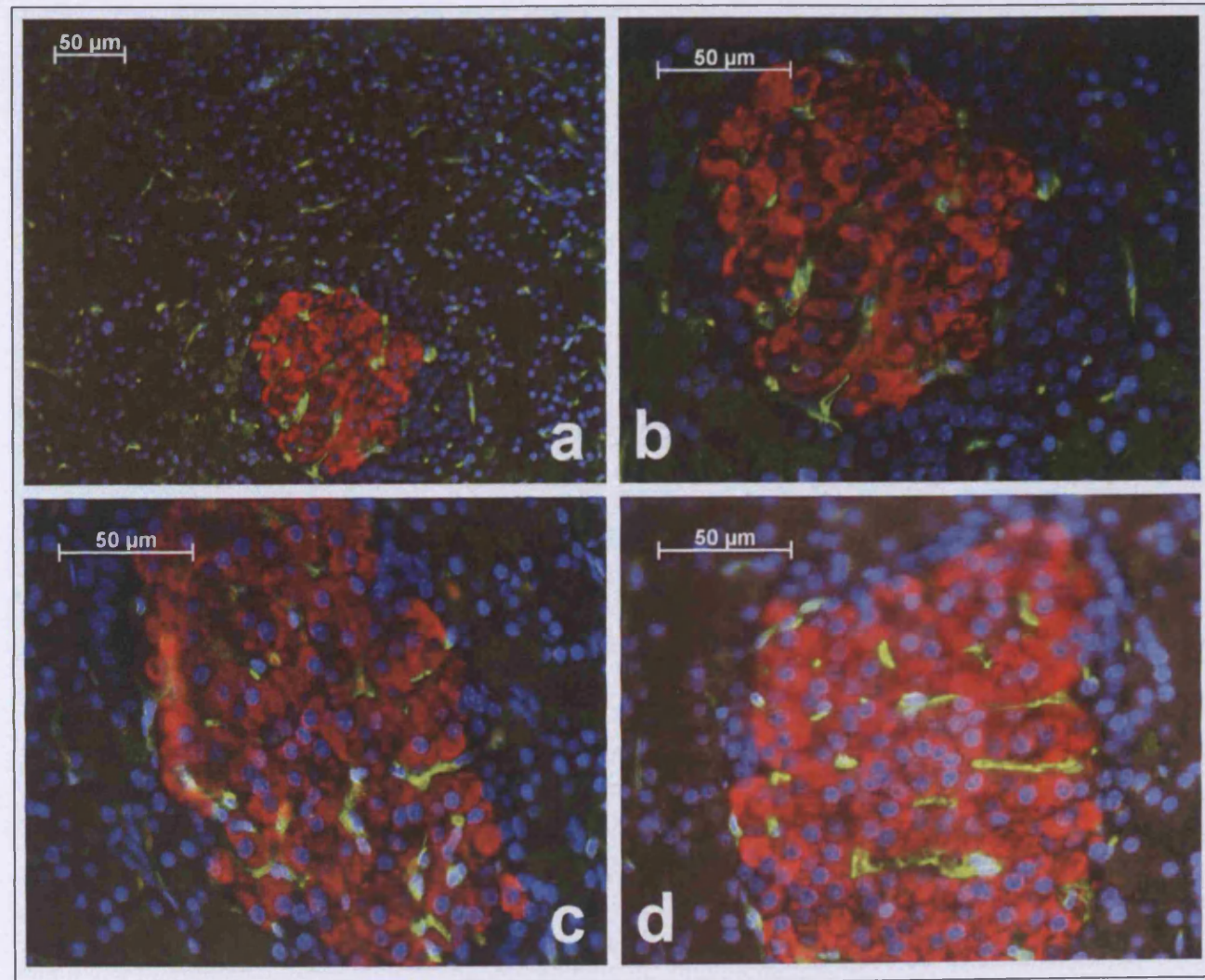
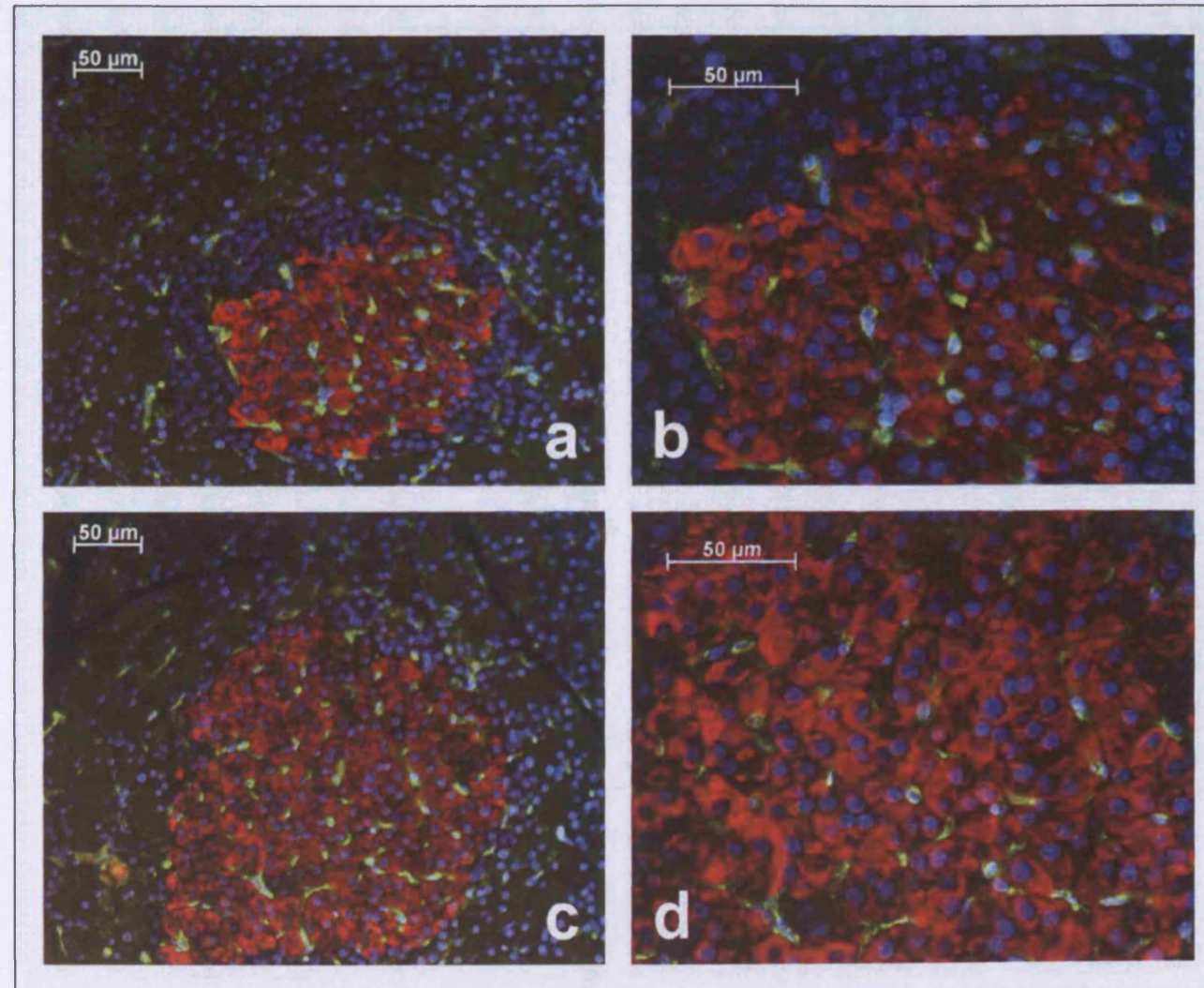


Figure 4.6: Large sized ($250\mu\text{m} +$) native islets within the rat pancreas. Image (a) and (b) are the same islet at 200x and 400x magnification respectively. Image (c) and (d) are the same islet at 200x and 400x magnification respectively.



4.4 Qualitative histological analysis of engrafting transplanted islets.

The original description of intra-portal islet engraftment suggested that islets revascularise in a sequential and coordinated fashion (Griffith, Scharp et al. 1977). The first stage of analysing engraftment was to examine the stained liver sections for gross morphological changes in islet structure, before trying to quantify the changes.

4.4.1 Recovery of islets from transplanted recipients

Right branch liver lobes from each recipient were divided into three separate blocks. The first block contained the entire right anterior lobe and second and third blocks contained the right posterior lobe, which had been divided in half antero-posteriorly. The tissue was sectioned at 4 μ m thickness, stained with the dual insulin and endothelium staining and analysed as outlined in Chapter 2. At least 10 islets were examined from each animal and subsequent deeper sections were cut from the blocks if there were insufficient islets visualised after analysing one section from each block. Only islets with an area over 1000 μ m² were used for analysis, in order to reduce bias of over/under representing endothelium.

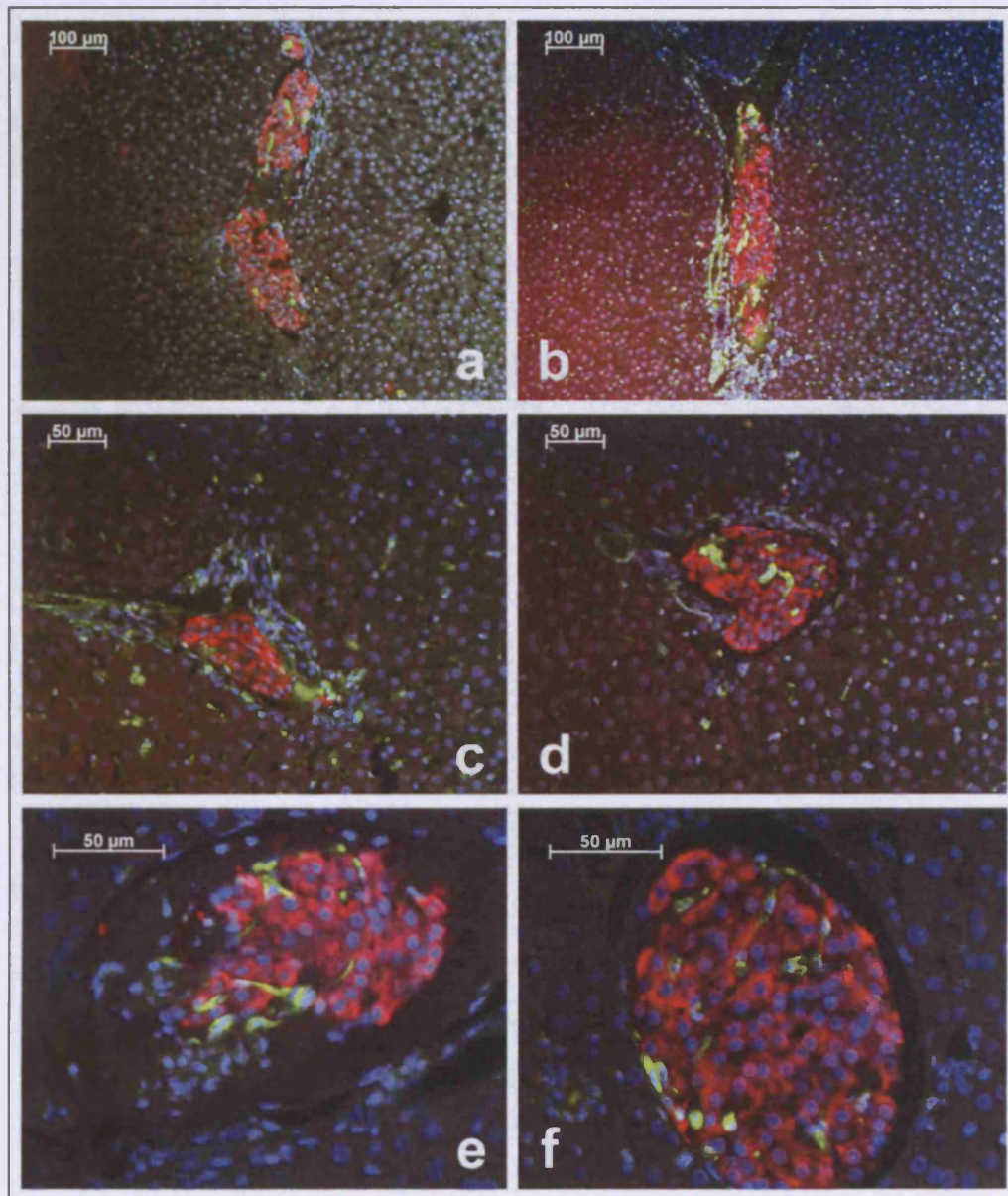
A total of 428 transplanted islets were analysed, with an average of 61.1 (\pm 3.1) islets per time point and 14.3(\pm 0.61) islets per animal. Each section contained average of 4.5 (range 0 to 14 islets per section) analysable islets with significantly less islets in sections from the right anterior lobes than the right posterior lobes; 3.0 islets per section in right anterior lobes, 5.8 islets per section ($p=0.001$) in the lateral division of the right posterior lobe and 4.7 islets per section ($p=0.018$) in the medial division of the right posterior lobes. This difference was thought to be flow mediated at the time of transplantation, as the branch of the portal vein supplying the right anterior lobe comes off the right branch of the portal at right angles and therefore limits flow to this lobe during transplantation.

4.4.2 The day of transplantation

On the day of transplantation, islets could be clearly visualised in the portal vein-figure 4.7. On the lower power sections (Figure 4.7 (a) and (b)), large islets can be seen to have embolised in the branches of the portal vein up to a point of bifurcation. The hepatic artery and a biliary duct can be clearly seen to the left of the islet in the lower part of figure 4.7b, confirming the location of the embolised islet to the portal triad. The size of the portal vein, in comparison to the surrounding artery and biliary duct, suggests some distension of the vein in response to the embolised islet, although the elongated shape of the islets would also suggest some compression of the islet architecture. The portal vein is completely occluded by the embolised islet and some sections show the early formation of thrombus within the vein. In figure 4.8a, the hepatic veins are clearly visualised either side of the embolised islet and the surrounding hepatic architecture is normal with no associated hepatic vacuolation.

Despite the compression of the islet, the general architecture is preserved. The insulin staining within the islet is bright and very little exocrine tissue is seen around the islet. The islets have bright endothelial staining and obvious vascular channels can be seen, which appear to be of a similar distribution and density to native islets.

Figure 4.7: Islets on the day of transplantation. All images are of different islets. Images (a) and (b) are at 100x magnification, (c) and (d) at 200x magnification and (e) and (f) at 400x magnification.

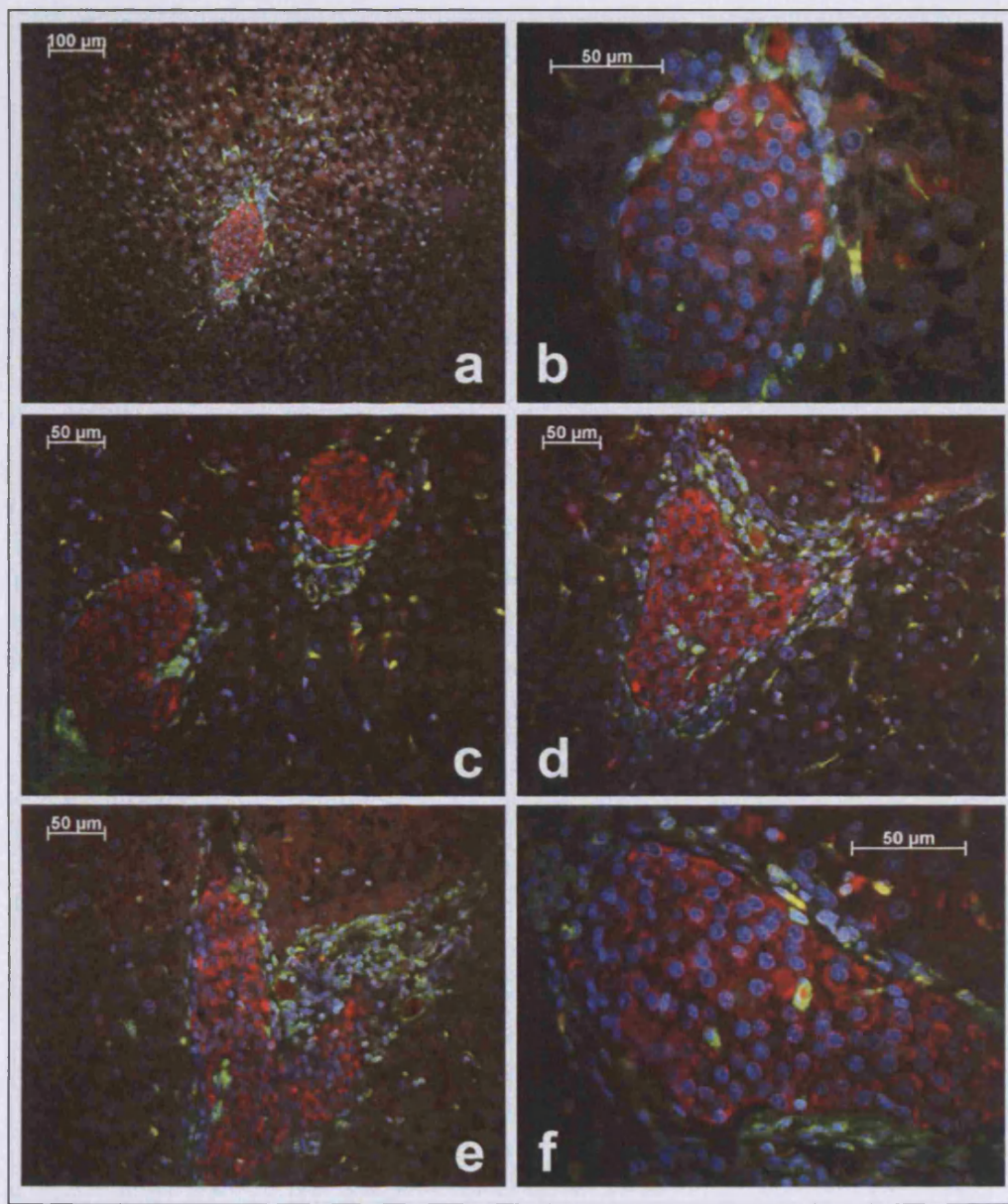


4.4.3 One day post transplantation

One day after transplantation, the islets are still fully occluding the branches of the portal vein- figure 4.8. The islets have not migrated out of the portal vein branches and are still surrounded by the endothelial staining of the venule walls. The lumens of the portal venules close to embolised islets are occluded by thrombus and there is no evidence of recannalisation. The liver architecture surrounding islets is now grossly vacuolated, as seen in figure 4.8a, although the liver structure does not appear to be distorted.

The insulin staining of islets appears bright and generally homogeneous, with no obvious areas of cellular death. The BS-1 staining within islets is generally sparse and most islets do not have evidence of stained endothelium. Of the islets that do have BS-1 staining, the pattern is similar to islets visualised on the day of transplantation. There is no evidence of endothelial invasion or revascularisation at this early time point.

Figure 4.8: Islets one day after transplantation. Images (a) and (b) are the same islet at 100x magnification and 400x magnification, (c) (d) and (e) are at 200x magnification and (f) is at 400x magnification.



4.4.4 Three days post transplantation

After three days post transplantation (figure 4.9), the islets have started to migrate into the hepatic architecture. The portal vein still has evidence of thrombus, although the portal vein has already recannilised in some places and has formed new endothelium over the surface of the islet, as seen in figure 4.9a and b. The hepatic architecture surrounding engrafting islets is still vacuolated but the rest of the liver architecture is normal with clearly demarcated hepatic arteries and veins.

The insulin staining within the engrafting islets is generally bright with good integrity of the beta cell mass, although some islets have areas which have lost insulin staining and appear degranulated- figure 4.9e. There are also areas of aggregated cells within the liver which have no insulin staining (figure 4.10) but appear to be embolised tissue, as seen by their close proximity to the portal triad (figure 4.10b). The cellular nuclei of these cells are swollen, which would suggest an element of necrosis. It is unclear whether the tissue is embolised islets which are necrotic or whether it is exocrine tissue which has been injected and embolised with the transplanted islets.

The engrafting islets (figure 4.9) have a paucity of vascular endothelium within the centre of the islet although there is endothelium around the outside of the beta cell mass. The circumferential endothelium is starting to form vessels (figure 4.9c and d) and endothelial cords can be seen invading into the beta cell mass of some islets (figure 4.9 b, d, e and f). These cords tend to be long unbranching structures and some appear to have double contouring, suggesting early blood vessel formation. Within the centre of the islets there appears to be areas of reduced staining between some of the insulin positive cells (figure 4.9c and f). It is unclear whether these may have been original native vascular channels which have been left devoid of endothelium or represent possible regression of beta cell mass.

Figure 4.9: Islets three days after transplantation. Image (a) and (b) are the same islet at 100x and 400x magnification. Images (c), (d), (e) and (f) are all different islets at 400x magnification. A hepatic arteriole (HA), hepatic ductule (HD) and portal venule (PV) are clearly visualised in the low power view. The surrounding hepatic stroma (HS) appears vacuolated. Non branching early vessels (EV) are clearly seen in some sections.

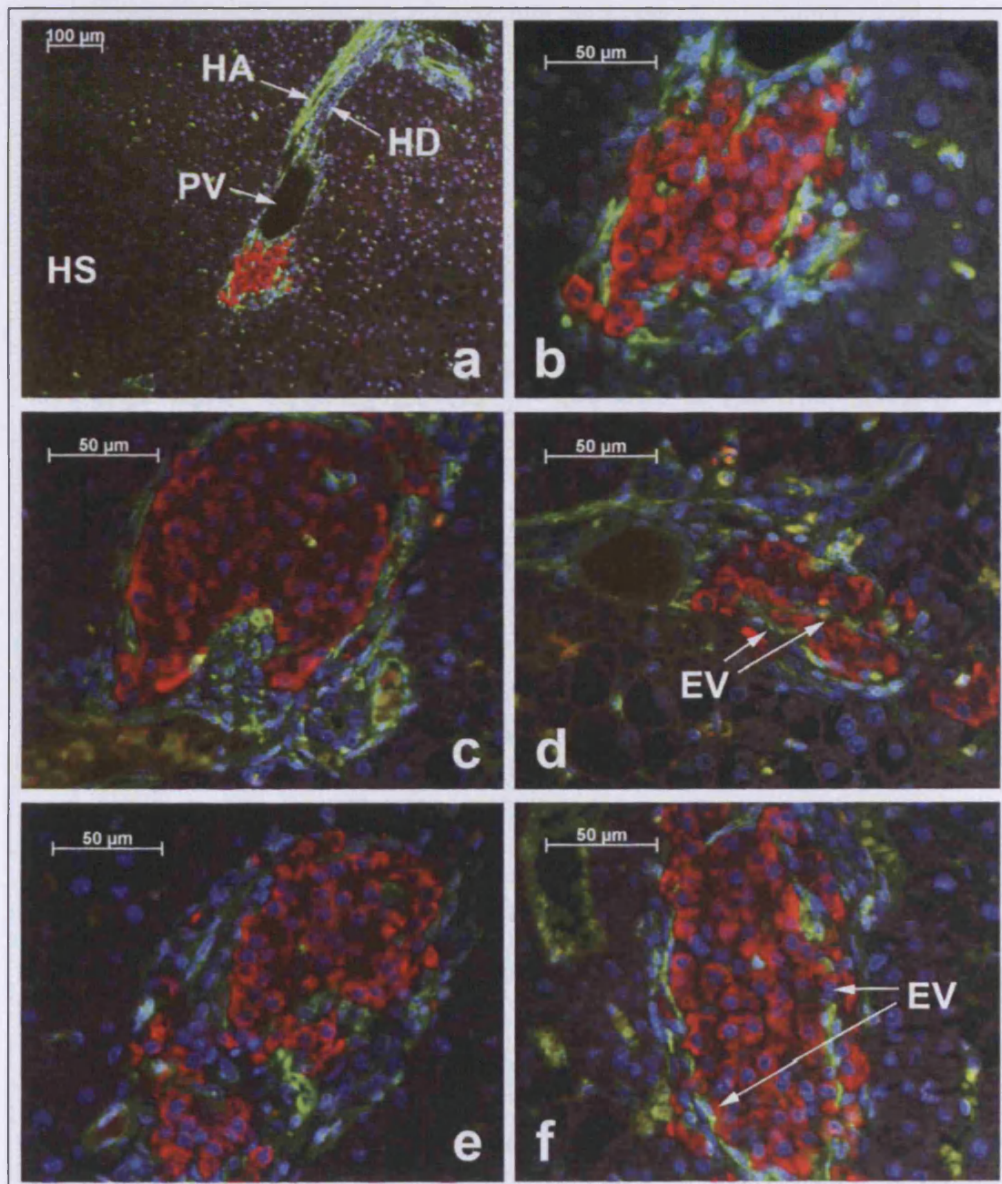
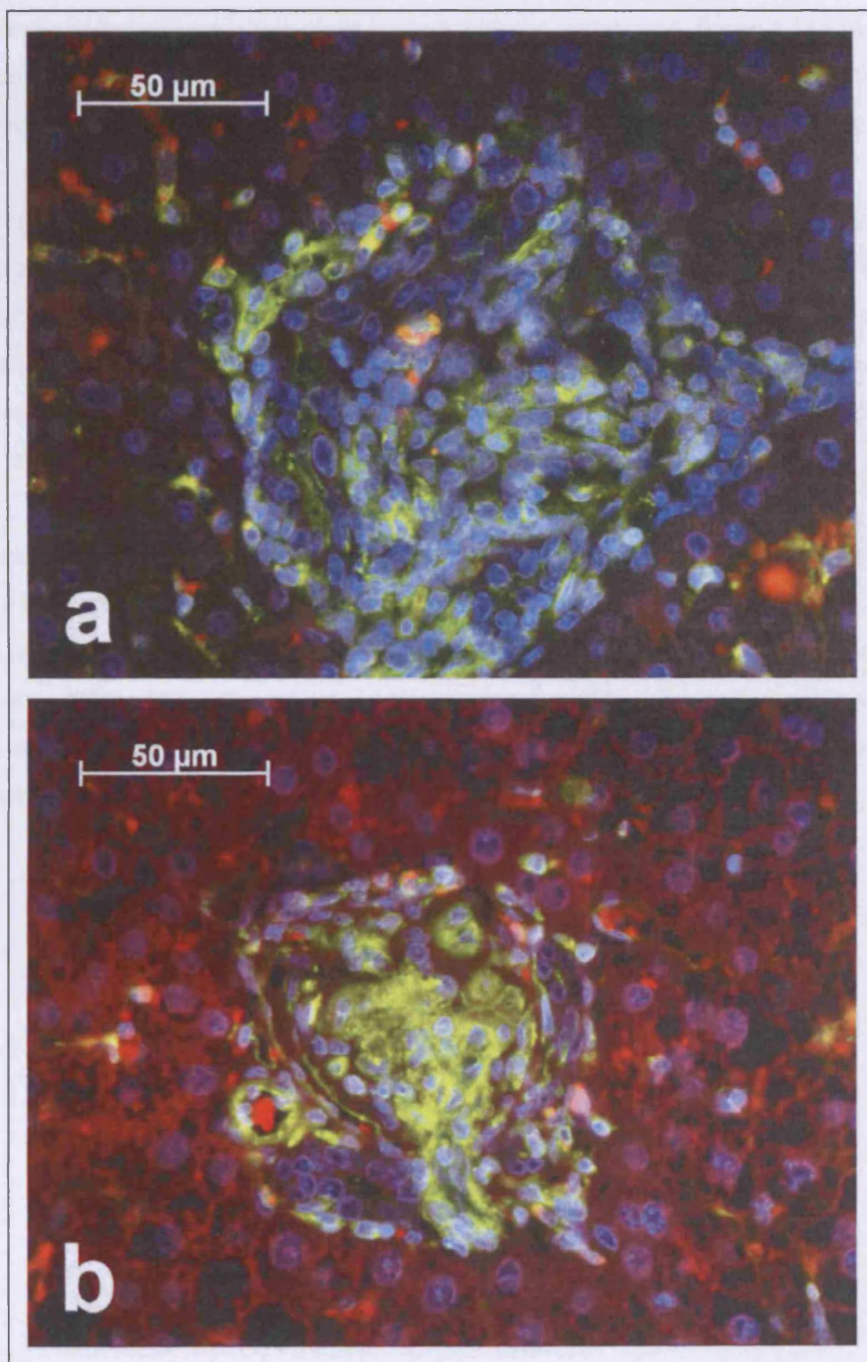


Figure 4.10: Liver sections 3 days after transplantation showing probable unengrafted islets. Both images are of different islets at 400x magnification.



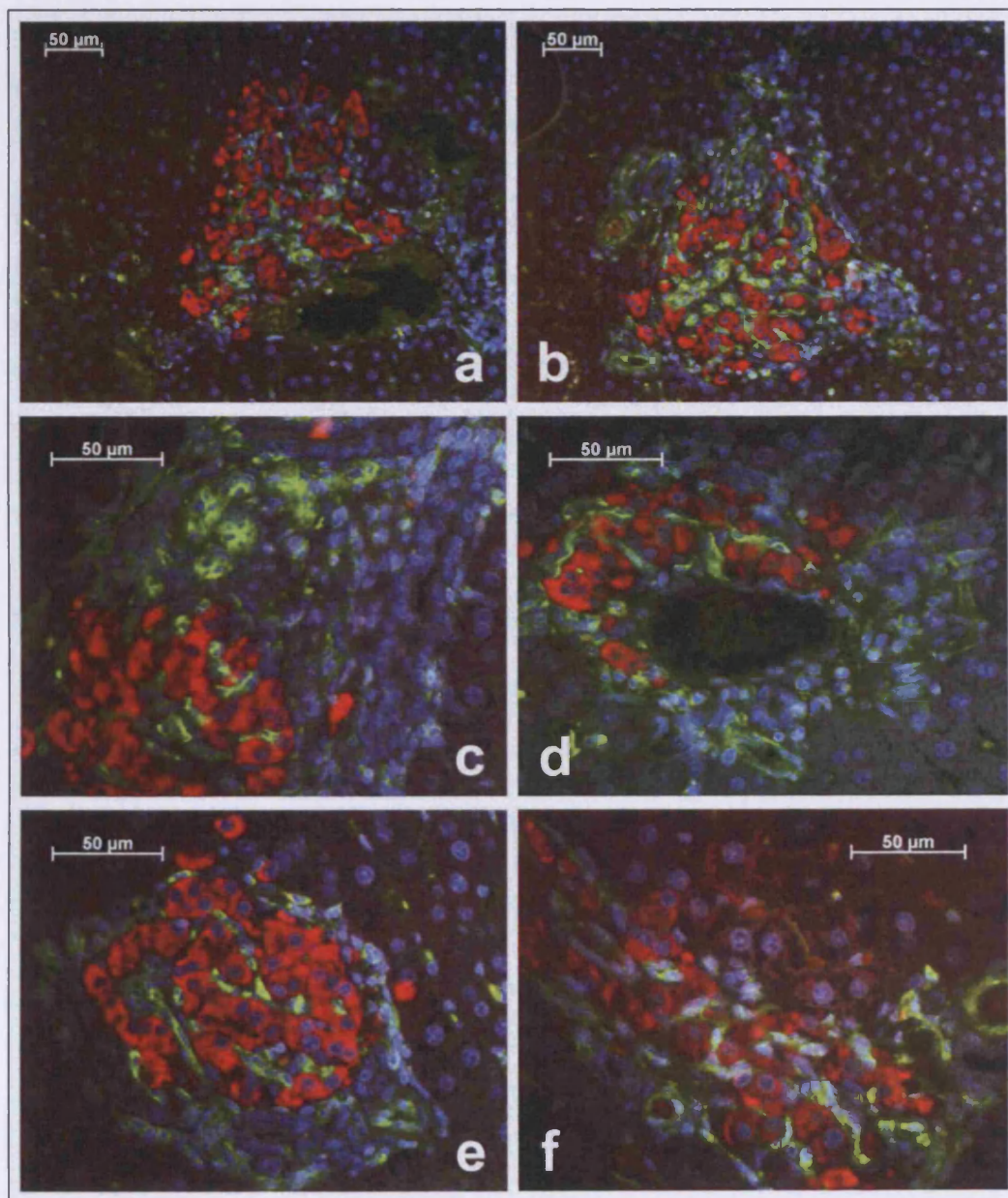
4.4.5 Five days post transplantation

Five days after transplantation (figure 4.11), the islets have migrated further into the hepatic tissue but still remain in close contact with the portal triad- figure 4.11a and b. The surrounding liver tissue looks less vacuolated and although the portal vein still has evidence of thrombus in places, recannilisation is well underway in a number of areas.

The endocrine portion of the islets now looks more heterogeneous with patchy insulin positive staining and larger gaps between insulin positive cells with the feeling of cell loss between islands of beta cells- figure 4.11d. Some islets have areas which are devoid of insulin staining and appear to be non viable (figure 4.11c). As seen at 3 days, there are also a number of areas of embolised tissue which are devoid of insulin and appear non viable (not shown).

The endothelial staining is now seen toward the centre of the islet and discrete vascular channels are discernable in some islets (figure 4.11f), although most BS-1 appears to be cords of cells. The pattern of staining has a branching architecture and an early network is starting to form in most islets. BS-1 positive staining is also seen within non insulin positive areas of the islet (figure 4.11c), although the staining appears to be random and it is not clear whether this represents endothelium or inflammatory cell infiltrate.

Figure 4.11: Islets 5 days post transplantation. All images are of different islets. Images (a) and (b) are at 200x magnification, images (c), (d), (e) and (f) are at 400x magnification.



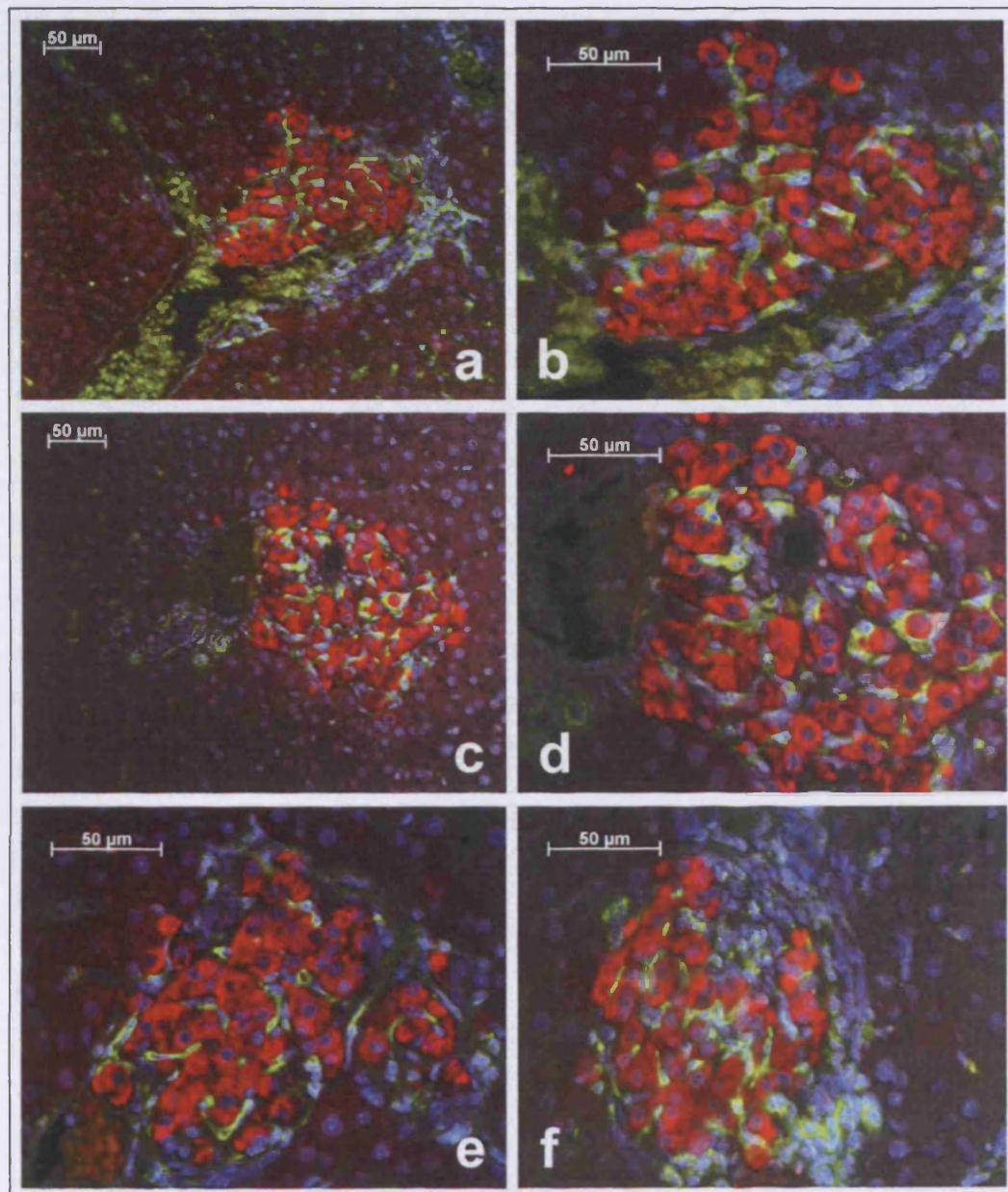
4.4.6 Seven days post transplantation

After seven days post transplant (figure 4.12), the islets have fully migrated into the hepatic architecture and remain in close contact with the portal triads- figure 4.12a,c and d. The orientation of the islets appears to be along the portal vein, instead of directing down the hepatic sinusoids, and most portal vein branches have recanalised. The surrounding hepatic tissue now looks normal and vacuolation is scarce.

The islets appear to be more organised than the day 5 islets, with smaller gaps between the insulin positive cells. The insulin staining within the islet is more homogeneous and staining is now bright. Some islets still have areas without insulin staining, although they are smaller and less frequent than day 5 islets- figure 4.12 e and f. In addition, the areas of non-viable cells with no insulin staining are scarce and small.

The endothelial staining has reached the centre of the islet and vascular channels are present toward the centre of the islets. The vascular channels have formed a network of vessels, although not all areas are fully organised.

Figure 4.12: Islets 7 days post transplantation. Images (a) and (b) are the same islets at 200x and 400x magnification, respectively. Images (c) and (d) are the same islets at 200x and 400x magnification, respectively. Images (e) and (f) are different islets, both at 400x magnification.



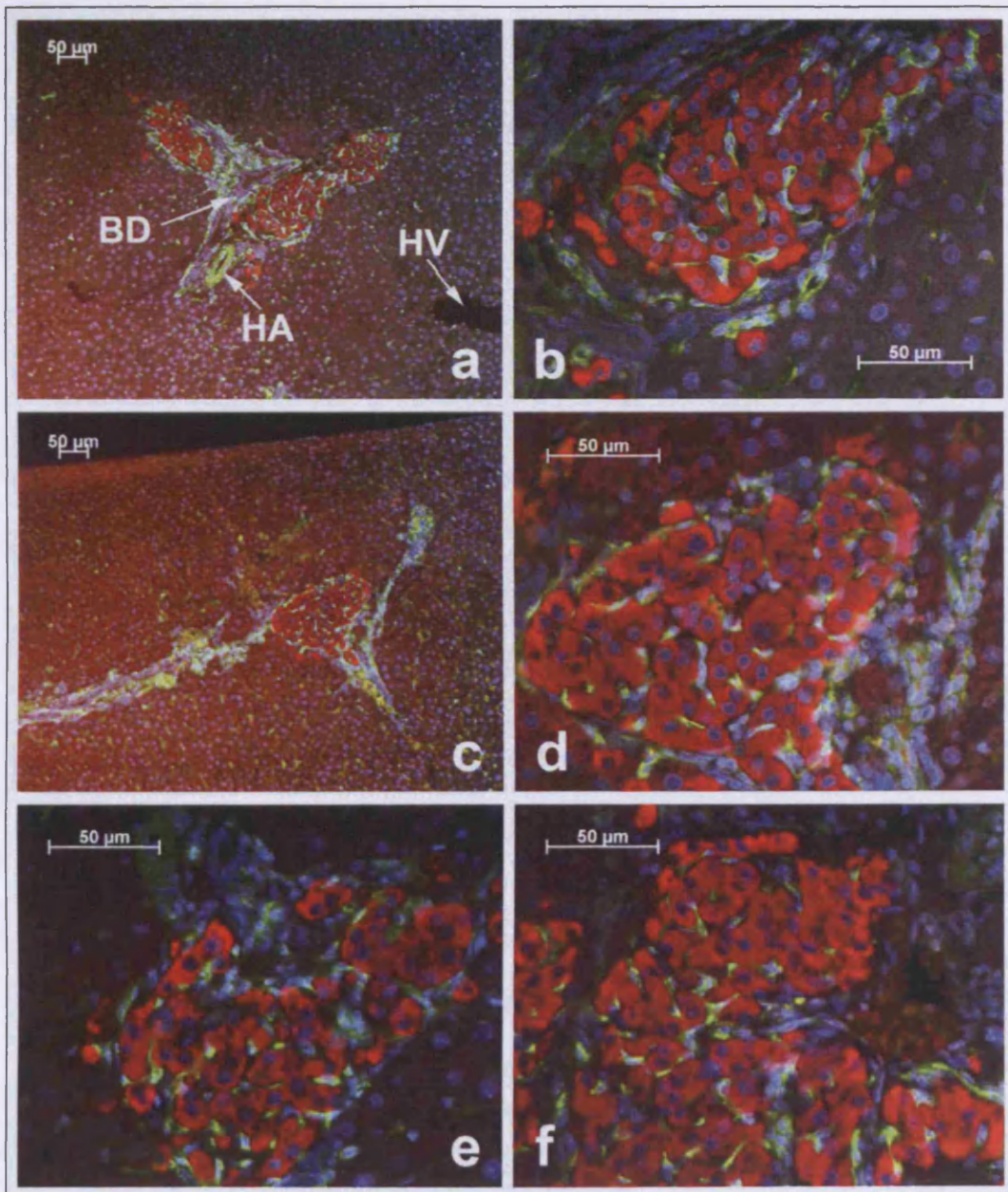
4.4.7 Fourteen days post transplantation

The islets appear to have fully engrafted by two weeks after transplantation (figure 4.13). Islets are still closely associated with the portal vein and are orientated in parallel with the direction of the portal triad (Figure 4.13a and 4.13c). The liver architecture is normal with some vacuolation of hepatocytes in areas close to the islets.

The beta cell mass of the islet is well defined with bright staining of the insulin positive cells and no areas of non insulin staining. The islets appear well organised and there are no gaps between the insulin positive cells. Interestingly, there is no rim of non staining cells, as seen in native islets, but there are a number of cells which are insulin positive just outside the well circumscribed area of the islet. It is not clear from this staining alone whether these are islet derived cells which have been stranded by dying beta cells around them or whether these are hepatocytes which are staining for positive insulin, either due to insulin uptake or phenotypic change.

The vascular architecture of the engrafted islets is complete with the reformation of a complex glomerular like vascular network throughout the islet. All the endothelial staining appears to be in the form of tubular like structures (endothelialised blood vessels) and covers the whole islet. The islets are smaller than native islets but the vascular density seems to be greater than that seen in the native pancreas.

Figure 4.13: Islets fourteen days post transplantation. Images (a) and (b) are the same islets at 100x and 400x magnification, respectively. Images (c) and (d) are the same islets at 100x and 400x magnification, respectively. Images (e) and (f) are different islets at 400x magnification. The low power view (a) shows a normal hepatic stroma with clearly visible hepatic arteriole (HA), bile ductule (BD) and hepatic venule (HV).

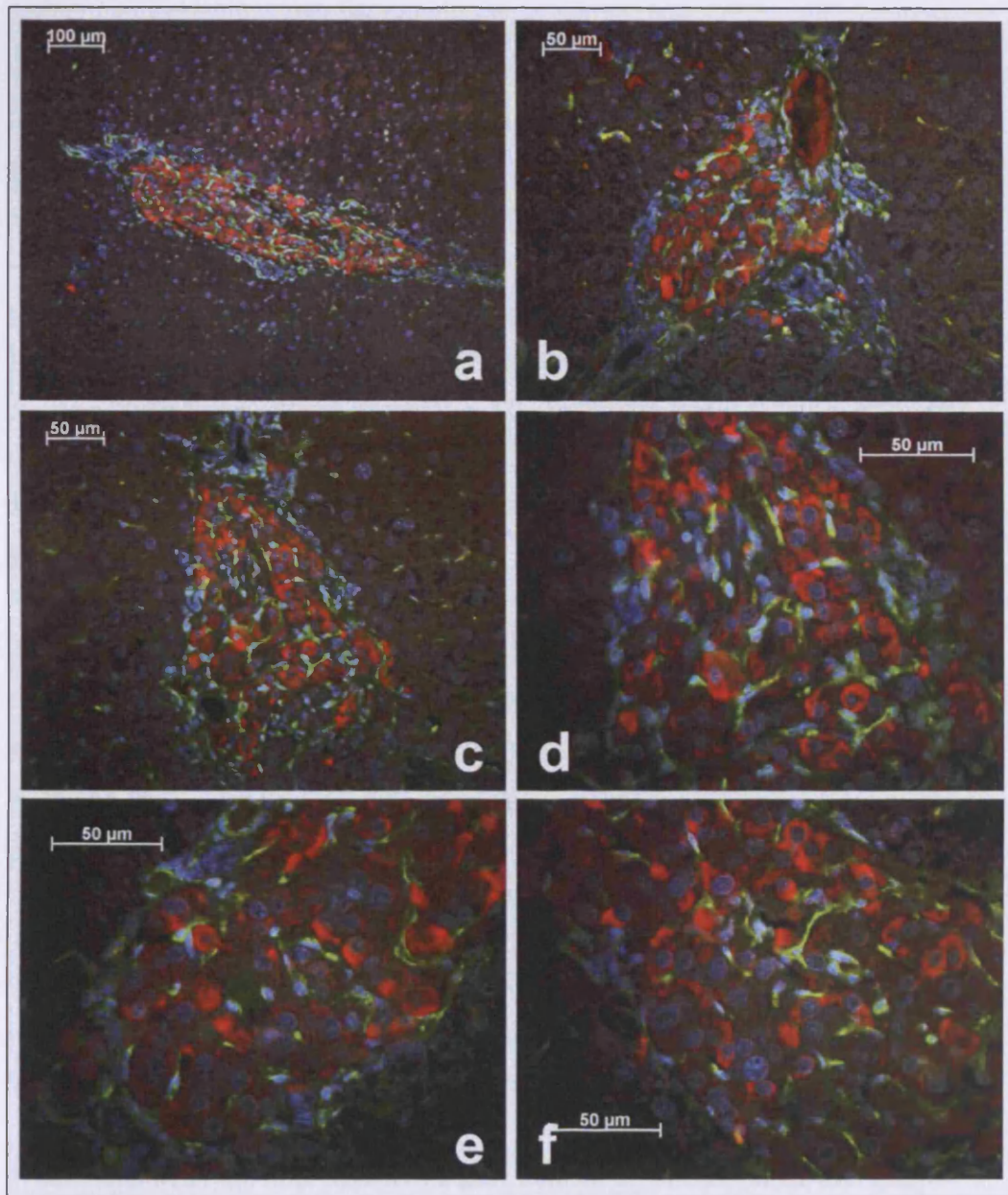


4.4.8 Thirty days post transplantation

After one month post transplantation (figure 4.14), the islets have changed very little since day 14 in their gross morphology. The islets are still closely associated with the portal triad (4.14a, b and c) and the surrounding hepatic architecture is normal with minimal vacuolation of hepatocytes.

The insulin staining is homogeneous and bright with obvious demarcation between hepatic tissue and beta cell mass. There are no areas of non insulin staining within the islet. The vascular architecture within islets is still a complex branching network and has not changed any further since day 14, although the network appears more extensive and vessels are smaller in calibre.

Figure 4.14: Islets thirty days post transplantation. Image (a) is at 100x magnification. Images (c) and (d) are the same islets at 200x and 400x magnification, respectively. Images (b), (e) and (f) are different islets at 400x magnification.



4.5 Quantitative analysis of native and transplanted islets.

The qualitative analysis of transplanted islets suggested that engrafted islets were smaller than native islets and that there was an obvious change in endothelial content and structure during the engraftment process. In order to develop a tool for analyzing modification of engraftment, these changes would have to be quantifiable and significant. Therefore, the images were analysed using image J software, as described in materials and methods, to see if there were any quantitative changes in the vascular architecture post transplantation.

4.5.1 Islet area in native and transplanted islets

The average area of islets was significantly reduced on the day of transplant when compared with native islets (figure 4.15) and slowly increased post transplant toward native levels. Although transplanted islets did not fully return to the area of native islets, there was no significant difference between transplanted and native islets after 7 days post transplantation.

The changes in islet area from one time point to the next were not significant but when changes were compared with the time point two places ahead, all changes were significant until day 7 was used as the reference. These results suggest that islets are smaller after transplantation, probably due to compression and loss of beta cell mass but remodel and increase in size until day 7 post transplant.

4.5.2 Percentage of islet comprised of endothelium

When compared to native islets, the percentage of the islets comprised of endothelium was significantly reduced on the day of transplantation and one day after transplantation but increased to a significantly higher level from day 3 onwards (figure 4.15).

The changes from one time point to the next were all significant except the change between day 7 and 14. In addition, the reduction in percentage vascular area from day 14 to 30 was also significant ($p < 0.001$).

4.5.3 Vascular density measurements

Both measurements of vascular density showed an initial fall after transplantation, followed by a rise to levels above that found in native islets. The two different methods did differ in the rate of increase in vascular density after transplantation, which reflects the nature of the vasculature formed early in the revascularisation process.

4.5.3a Branch counting method

Using the branch counting method, the vascular density was $1358(\pm 50)$ vessels per mm^2 in native islets and fell immediately after transplantation to reach a nadir on the first day post transplantation before increasing to values above native islets on day five and progressively increasing up to day 30 post transplantation. See figure 4.16. The increases in vascular density between time points were statistically significant except between day 5 and 7, and day 14 and 30.

4.5.3b Grid method

This pattern of vascular density change with the grid method was similar to the vessel branching method but the increase of vascular density after day 1 was more rapid- figure 4.16. This method calculated the vascular density to be $1826(\pm 73)$ vessels per mm^2 in native islets which immediately fell post transplantation and reached a nadir on the first post transplant day. The density subsequently rose rapidly to achieve values above that found in native islets by day three post transplantation and continued to increase until day 5. After day 5, the vascular density did not change significantly. The sequential changes between time points were significant up to day 5 and subsequent changes were not significant.

Figure 4.15: Quantitative changes in average islet area (μm^2) and percentage of islet comprised of endothelium during engraftment. Values are measured in native islets, islets on the day of transplantation and day 1, 3, 5, 7, 14 and 28 post transplantation. Values are expressed as means plus standard error. The changes in values were compared to native islets and p values less than 0.01 are indicated on the graph by a *.

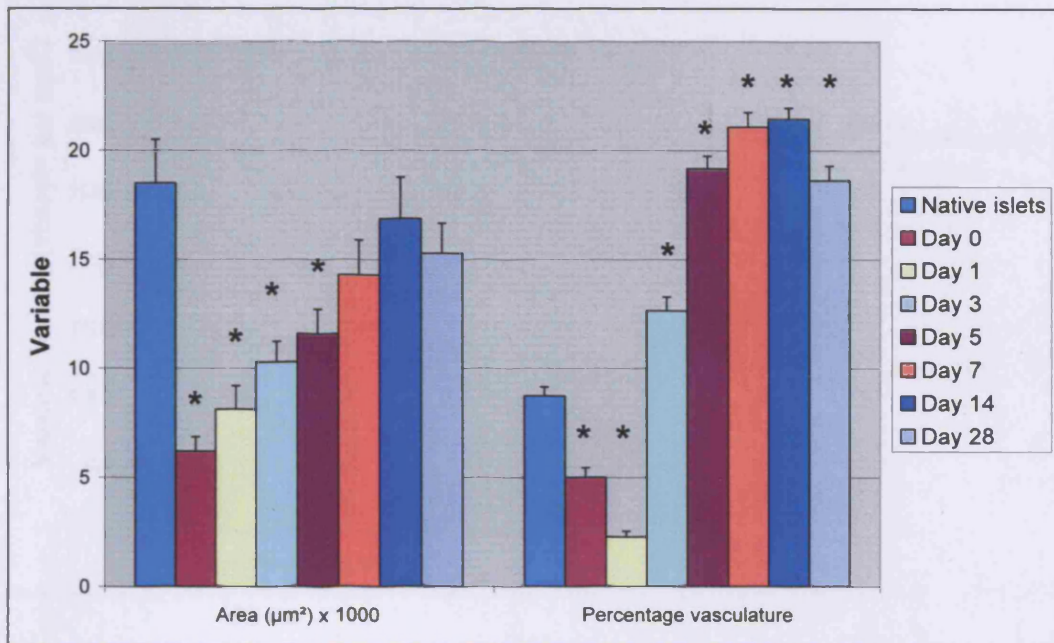
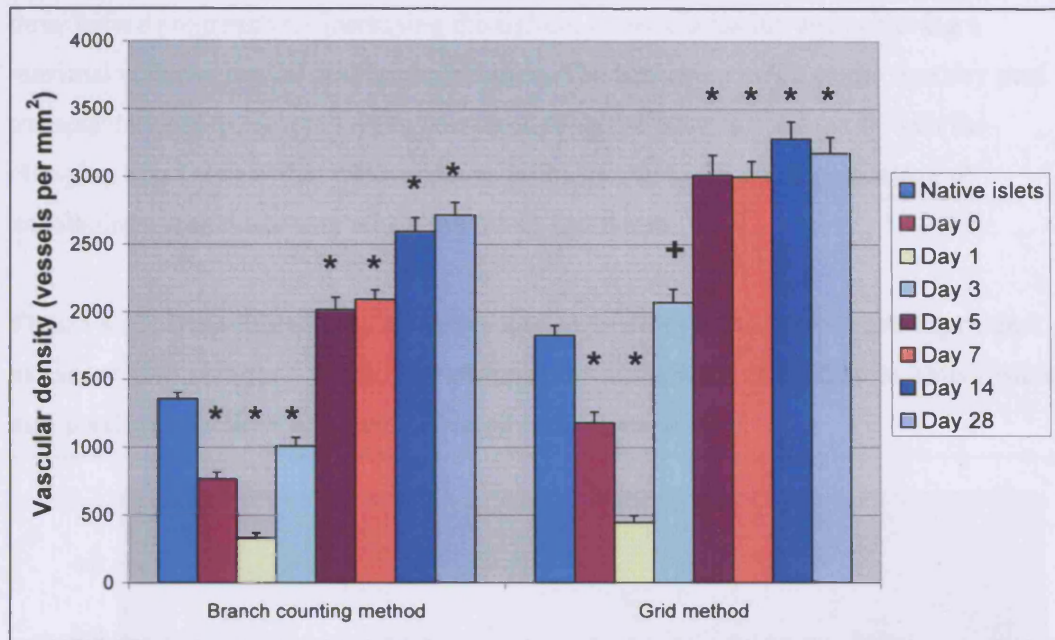


Figure 4.16: Quantitative changes in islet vascular density during engraftment.
Values are measured in native islets, islets on the day of transplantation and day 1, 3, 5, 7, 14 and 28 post transplantation. Values are expressed as means plus standard error. The changes in values were compared to native islets and p values less than 0.01 are indicated on the graph by a * and p values less than 0.05 by a +.

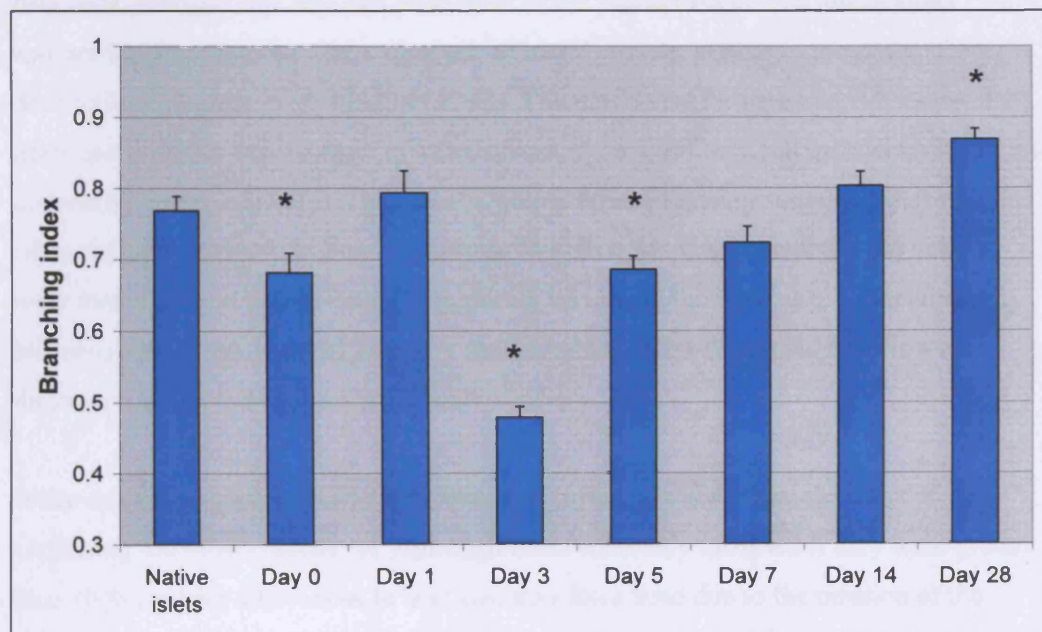


4.5.4 Branching index

The branching index was used to quantify the relative branching of vessels within islets and whether a new glomerular type network was formed within the islet- figure 4.17.

The branching index fell from $0.77(\pm 0.02)$ in native islets to achieved a nadir on day three before progressively increasing throughout revascularisation and achieving a maximal value on day 30 post transplantation. The branching index on the first day post transplantation was elevated when compared to native islets but did not fit with the changing trend seen within islets and was probably due to the small percentage of endothelium within islets on which the result was based.

Figure 4.17: Branching index in native and engrafting islets. Values are expressed as means plus standard error. The changes in values were compared to native islets and p values less than 0.01 are indicated on the graph by a *.



4.6 Discussion

The current model used for islet transplantation worked well with good resolution of clinical parameters post transplantation and easy histological retrieval of islets from recipient livers. The staining of islets within the pancreas and liver sections provided good delineation of β -cell cell mass with clear identification of endothelium within native and transplanted islets. The analysed images generated from qualitative histological analysis subsequently provided further quantitative evidence for the stepwise progression of islet revascularization. These observations and the implications relating to islet revascularization will now be considered.

4.6.1 Assessment of native islets

The native pancreatic islet architecture was well visualised with the dual staining technique and comprised of a central core of beta cells with a surrounding mantle of non beta cells, although this was less prominent in the smaller islets. The native islets appeared highly vascular with a network of blood vessels, similar to previous descriptions (Bonner-Weir and Orci 1982). The blood vessels appeared to traverse the islets and coalesce in a network of vessels surrounding the beta cell mass or outer mantle, depending on the islets size. The gross histological examination suggested that smaller islets did not have an extensive glomerular like network of blood vessels but vessels were more likely to be non-branching, purely traversing the islet core. Unfortunately, this observation was not reinforced by the branching index which did not show any difference between large and small native islets.

When considering the vascular architecture in different sized islets, the issue of sectioning has to be considered. Although islets were only analysed if they were greater than $1000\mu\text{m}^2$, the differences in islet size may have been due to the position of the section in relation to the islet being examined. If islets are considered to be almost spherical in shape and a large islet was sectioned at the very periphery of the sphere, the sectioned islet would appear to have the same area as a smaller islet that was sectioned through the centre of the sphere. This situation would confound analysis of the vascular architecture of different sized islets because it would be difficult to differentiate between

small islets and the edge of large islets. Serial sections may have obviated this confounding factor but were not used for the analysis. The random orientation of islets within cut sections should have provided arbitrary samples through all parts of islets and, given the number of islets analysed, the sample size would have reduced the confounding factor of how islets were sectioned. The problem of sectioning through islets would only have affected the observation of small islets, as large areas could only have arisen from large islets. Lastly, the observation of different vascular anatomy in association with the size of native islets has been reported before by a number of different authors (Bonner-Weir and Orci 1982; Lifson, Lassa et al. 1985; Brunicardi, Stagner et al. 1996) and the observations reported here are in keeping with previous publications. The sectioning of islets may have been a confounding factor for examining the change in vascular architecture with size of islets and the model could have been further improved by the use of serial sections or thicker sections with the use of scanning confocal microscopy.

4.6.2 The change in islet area after transplantation

Islet area changed dramatically after transplantation with an immediate fall on the day of transplantation which was followed by a gradual rise to levels just below the area of native islets by day 14. Although some of the reduction in islet size may be explained by the loss of islet cell mass during isolation from the whole pancreas, the reduction in islet area of over 50% would be out of keeping with that observed in freshly isolated islets in culture. The force of embolising an islet into portal venules might have played a part in reducing the size of the islet, as free islets are pushed into ever decreasing sized vessels until forward motion is stopped by the occlusion of the vessel. Unfortunately, this effect is also minimal and the change in islet size after transplantation may be better explained by sectioning artifact. This situation would arise when a spherical islet is forced into a portal venule and made to take on the cylindrical shape of the vessel. Although islets were visualised within vessels in cross section (perpendicular to vessel direction) and along their long axis (within the same plane as the vessel direction), random sectioning is more likely to examine islets in short axis (cross section) than in long axis. This situation would have biased the measurement of early post transplant islets toward

smaller cross sectional islets and therefore reduced the area of islets sampled at this time point. With time, the islets are integrated into the hepatic architecture and probably take on a more spherical shape which would reduce the variability of directional sectioning and may explain the increase in islet area over time. Despite the changes in islet area after transplantation, the percentage vasculature and vascular density are unlikely to be affected as both measurements are calculated per unit area and would not be affected by orientation of sectioning.

4.6.3 The revascularisation of transplanted islets

The vascular architecture of islets changed markedly after transplantation in a stepwise and logical fashion which started with regression of native endothelium. On the day of transplantation endothelium was clearly seen within the centre of the islets although the staining appears sparser than in native islets. This observation is supported by the lower percentage vascular area and vascular density at this time point and suggests that even by the time of transplantation, and probably due to the isolation process, islets have already started to lose endothelium. On the first post transplant day, there is very little endothelium within the islet suggesting that further regression/differentiation of endothelium has occurred with no evidence of revascularization. By day three, islets have a paucity of endothelium in the beta cell core but endothelial staining is increased at the periphery of the islets and there are discernable endothelial cords which appear as single, non branching vessels traversing outer parts of the islet. This observation corresponds to a low branching index found at day 3 and suggests that the first step of revascularization is the piercing of the islet by single vessels which subsequently branch to form a network of vessels. The subsequent branching of these vessels corresponds to the gradual increase in branching index and vascular density found between day 3 and day 30.

By day five, endothelium is seen invading deeper into the islet and discrete vessels are starting to form. The vascular density, measured by the grid method, has increased from day 3 but further increases are small. These observations suggest that the network of endothelial cells has probably formed as early as day 5 and after this time point, the

tubular formation of vessels and branching to form a glomerular network occurs. By day seven, there are discernable vessels in the centre of the islet. The percentage vasculature has increased only a small amount from day 5 and subsequent increases are not significant. There has been no change in vascular density but subsequent increases occur after this time point in association with increases in the branching index. Therefore, endothelium is laid down at an early stage and after day 5 post transplantation, the transformation into a vascular network takes place. These observations correlate well with the previous in-vivo description of islet revascularization where one or two perforating afferent vessels enter the islet during the early stages of revascularization but the vascular density increase and flow of blood cells through vessels is only seen after day 6 (Vajkoczy and Menger 1995; Vajkoczy, Menger et al. 1995). This would suggest, as with other examples of neovascularisation, that the initial endothelial structure is laid down early (up to day 5) and is followed by a quiescent phase of perivascular cell recruitment and tubularisation of endothelium occurring between day 5 and day 7.

The measured parameters of vascular density and branching index changed very little from day 5 to day 7 but increased significantly between day 7 and 14 post transplantation with further significant changes of the branching index occurring out to day 30. Although formed vascular channels were visualized at day 7 post transplant, further remodeling of the vascular network appears to take place after this time point. It is therefore difficult to suggest exactly when the islet is fully revascularised as a rudimentary vascular network appears to be in place by day 7 post transplant but further changes are still occurring by day 30 and some authors have suggested that remodeling continues long after this time point (Rooth, Dawidson et al. 1989). These experiments were not continued after 30 days and it is therefore impossible to say whether further remodeling occurs after this time point.

When considering this model as a tool for assessing the effects of accelerating revascularization, the late remodeling changes are probably irrelevant and the important time point is probably between day three and seven after transplantation. This early time point is critical as maximal graft loss occurs around day 3 (Davalli, Scaglia et al. 1996)

and early functional vessels are not seen until day 6 (Vajkoczy and Menger 1995; Vajkoczy, Menger et al. 1995). Therefore, any successful manipulation of revascularization would have to allow the beta cell mass to last longer or the revascularization to occur at an earlier point and reduce hypoxic cell death. The observation and quantification produced by this model changed at its fastest rate during this time period and would therefore be sensitive enough to detect any change in the time course of revascularization associated with islet manipulation. Therefore, observation and quantification of islet vascularisation between days 3-5 should produce a robust model for assessing the manipulation of islet revascularization and engraftment.

4.7 Conclusions

These experiments have shown that the selective right branch intra-portal transplant model with subsequent dual BS-1 and insulin staining provides a robust model for the study of islet revascularization. The qualitative and quantitative analysis suggests that transplanted islets revascularise in a coordinated and sequential fashion which starts with an initial reduction in endothelial content and progress from invasion of new endothelium through to formation of a completely new vascular network. The revascularization process can be visualised as early as day 3 and is probably complete by day 7 but vascular remodeling continues long after revascularization is complete.

Chapter 5 – Results:

In-vitro desferrioxamine treatment studies

5.1 Introduction

The experiments described in the previous chapter have shown that the revascularisation of transplanted islets is a complex and orderly process. The coordination of this intricate process requires the sequential expression of a number of growth factors (Vasir, Reitz et al. 2000) in order to achieve the angiogenic stimulus necessary for islet revascularisation. The manipulation of some of these growth factors has been shown to improve clinical outcomes in islet transplant models and may enhance the survival and function of islets (Nakano, Yasunami et al. 2000; Garcia-Ocana, Takane et al. 2003; Lopez-Talavera, Garcia-Ocana et al. 2004). Unfortunately, the techniques employed require either genetic modification of islets or local delivery of factor to provide their effect. The treatment of islets with desferrioxamine potentially obviates these complications and provides a simple technique for manipulating angiogenic growth factors which would be easily transferable to the clinical setting.

Desferrioxamine was originally discovered as a by product of antibiotic research and has been available for the treatment of iron overload since the early 1960s (Hershko, Konijn et al. 1998). The clinical benefit of desferrioxamine is achieved by chelation of intracellular iron and subsequent removal of iron from the body by urinary excretion. Most intracellular iron is bound to transferrin and only about 5% is freely available in the labile iron pool (Kakhlon and Cabantchik 2002). It is this pool of iron which is available for cellular functions and is chelated by desferrioxamine. The removal of iron from the labile pool has a number of effects and iron depletion with desferrioxamine has been shown to up regulate HIF-1, inhibit the enzyme ribonucleotide reductase (Cooper, Lynagh et al. 1996), precondition myocardium (Dendorfer, Heidbreder et al. 2005) and brain (Prass, Ruscher et al. 2002) to ischaemic damage and alter the expression of important cyclins necessary for cell cycling (Le and Richardson 2002). The concentration of DFO required to achieve these in-vitro effects is higher than those used

clinically (~5 μ M) (Cooper, Lynagh et al. 1996) and most authors have used DFO concentrations of 100 μ M (Ebert, Gleadle et al. 1996; Yamakawa, Liu et al. 2003) and 130 μ M (Wang and Semenza 1993) to induce HIF-1. Desferrioxamine concentrations of 25 - 300 μ M (Cooper, Lynagh et al. 1996) have also been used to study the modulation of ribonucleotide reductase, with doses of 100 μ M causing an 80% reduction in DNA synthesis (Zanninelli, Glickstein et al. 1997). The dose of DFO used here was based on the experience of these authors and 100 μ M DFO was chosen as an experimental dose, with ten fold dilutions above and below for comparison of effect.

The initial in-vitro desferrioxamine treatment experiments were designed to explore whether DFO would up-regulate the expression of important angiogenic factors. The candidate growth factors were chosen either for their previously reported importance during islet revascularisation, VEGF, HGF and bFGF (Gorden, Mandriota et al. 1997; Vasir, Aiello et al. 1998; Vasir, Reitz et al. 2000; Vasir, Jonas et al. 2001), or their ability to improve post transplant outcomes when up regulated, VEGF and HGF (Garcia-Ocana, Takane et al. 2003; Lopez-Talavera, Garcia-Ocana et al. 2004; Lai, Schneider et al. 2005; Schultz, Fanburg et al. 2006). Angiopietin 1 was chosen for its ability to stabilise vasculature and work synergistically with VEGF (Davis, Aldrich et al. 1996; Thurston, Rudge et al. 2000). The expression of an important angiogenic inhibitor, endostatin (O'Reilly, Boehm et al. 1997), was also explored in order to see whether DFO treatment could suppress the expression of this inhibitory molecule and improve angiogenesis. Once the growth factor effects were determined, further in-vitro experiments were performed to investigate whether DFO altered islet viability and function, in view of the cell cycling modulation induced by iron chelation. This chapter will describe the results of these experiments and discuss the underlying mechanisms for the associated changes.

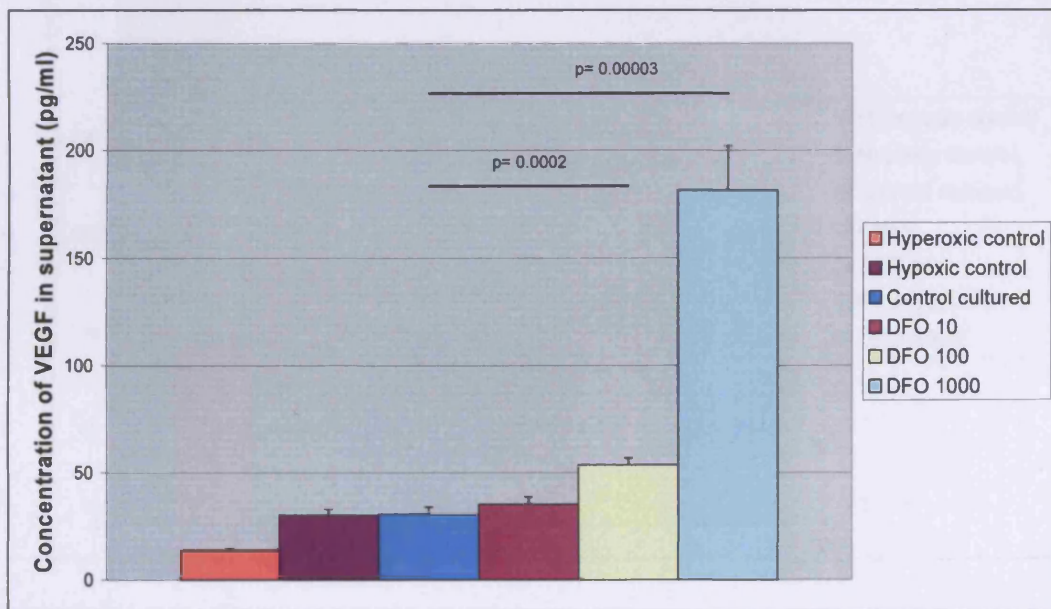
5.2 Effect of desferrioxamine treatment on protein expression of growth factors

These experiments used in-vitro culture techniques to explore whether desferrioxamine would alter the protein expression of important angiogenic growth factors. The number of growth factors explored by this technique was limited by the availability and sensitivity of suitable ELISA kits and further growth factor analysis was performed using molecular techniques for analysis of growth factor messenger RNA.

5.2.1 VEGF protein expression from desferrioxamine treated islets after overnight culture

Overnight incubation of islets with DFO produced a dose-dependent increase in mean supernatant VEGF protein concentration when compared to islets cultured in CMRL alone (figure 5.1): 30.6pg/ml control cultured, 35.3pg/ml DFO 10 μ M ($p=0.14$), 53.5pg/ml DFO100 μ M ($p<0.001$) and 181.5pg/ml DFO 1000 μ M ($p<0.001$). Supernatants from islets incubated in 1% oxygen did not have higher concentrations of VEGF when compared to control cultured supernatants: 29.9pg/ml hypoxic control ($p=0.30$). Supernatants from islets incubated in 95% oxygen had significantly lower concentrations of VEGF, compared to control cultured supernatants: 13.8pg/ml hyperoxic control ($p=0.004$). Ten sets of cultures were analysed in each group.

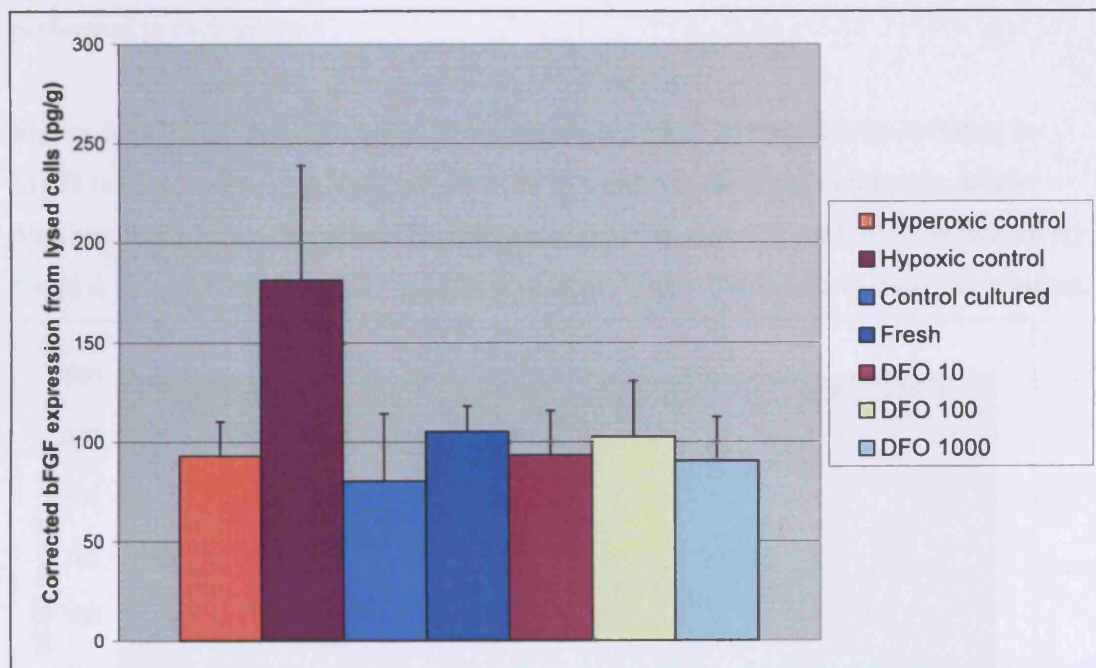
Figure 5.1: VEGF concentration in supernatants from islet cultures after overnight incubation in increasing concentrations of desferrioxamine (DFO 10 = desferrioxamine 10 μ M, DFO 100 = desferrioxamine 100 μ M and DFO 1000 = desferrioxamine 1000 μ M). Values are expressed as mean plus standard error.



5.2.2 bFGF protein expression from desferrioxamine treated islets after overnight culture

Unlike the VEGF experiments, there was no significant difference in bFGF protein expression after overnight culture with DFO when compared to control cultured islets (figure 5.2): 80.4pg/g control cultured, 93.4pg/g DFO 10 ($p=0.30$), 102.7pg/g DFO 100 ($p=0.23$) and 90.40pg/g DFO 1000 ($p=0.34$). The expression of bFGF in fresh (105.2pg/g, $p=0.17$) and hyperoxic control islets (93.0pg/g, $p=0.28$) was not different from control cultured islets but islets cultured in 1% oxygen had a significantly higher expression of bFGF protein (181.4pg/g $p=0.034$), when compared to control cultured islets. Six sets of cultures were performed in each group.

Figure 5.2: Corrected bFGF concentration from 350 lysed islets after overnight culture in increasing concentrations of DFO or lysed fresh islets. Values are expressed as mean concentration of bFGF protein per g of total protein from cell lysates plus standard error.

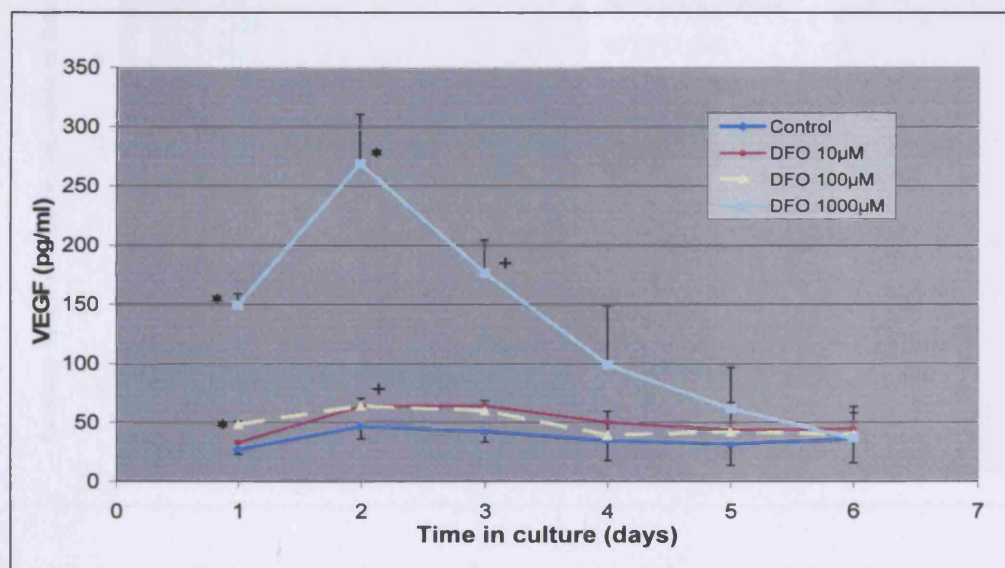


5.2.3 VEGF protein expression from desferrioxamine treated islets after culture in M199

The overnight incubation of islets with DFO produced a dose dependent increase in VEGF expression but it was unclear how long the increased expression would be sustained and whether VEGF expression would rapidly return to basal levels after transfer to an iron-containing environment. The time course of VEGF expression induced by DFO was examined by culturing islets overnight with DFO and subsequently transferring them to an iron containing media. This experiment was designed to simulate a transplant procedure where DFO cultured islets would be transplanted into the portal system and bathed in iron containing blood.

This experiment showed that VEGF protein levels remained significantly elevated in the supernatants of islets that were initially treated with DFO1000 μ M and DFO100 μ M for 48 and 24 hours respectively after transfer into iron-containing media, when compared to supernatants from control cultured islets (figure 5.3). Six sets of cultures were performed in each group.

Figure 5.3: VEGF concentration in supernatants from extended islet cultures in M199 medium after overnight incubation in desferrioxamine. Values are means plus standard error. Statistical significance: p value of less than 0.01 is indicated by * and a value of less than 0.05 is indicated by +, when compared to control cultures.



5.3 Effect of desferrioxamine treatment on gene expression of growth factors

One of the rationales for treating islets with desferrioxamine was to over express a number of angiogenic growth factors by a more physiological method than adenoviral gene transfection techniques. The previous experiments showed VEGF protein expression increased after treatment with DFO but ELISA protein assays were not available for a number of other rat specific factors. Therefore the expression of a number of important growth factors was explored using mRNA techniques.

The increase or reduction in expression was compared to fresh and control cultured islets, as outlined in figure 5.4 and figure 5.5 respectively. The experiments were performed on 5 sets of islets with duplicate analysis of expression.

Figure 5.4: The effect of desferrioxamine treatment on the expression of angiogenic growth factor RNA expression, compared to fresh islets. The values are presented as average fold change plus standard error. Statistical significance: p value of less than 0.01 is indicated by * and a value of less than 0.05 is indicated by +, when compared to control cultures.

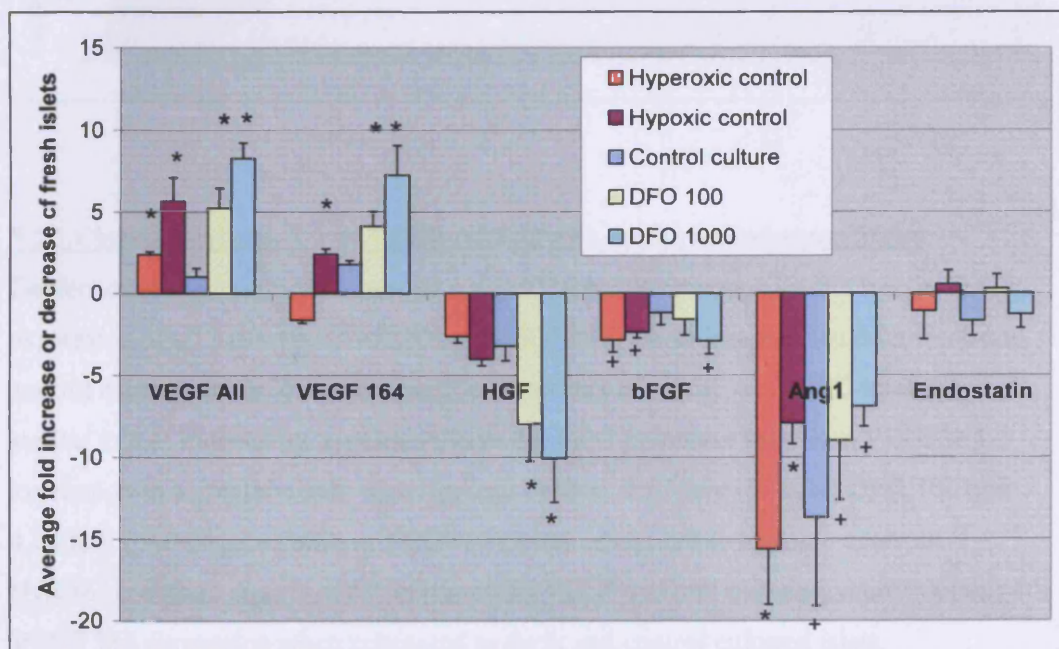
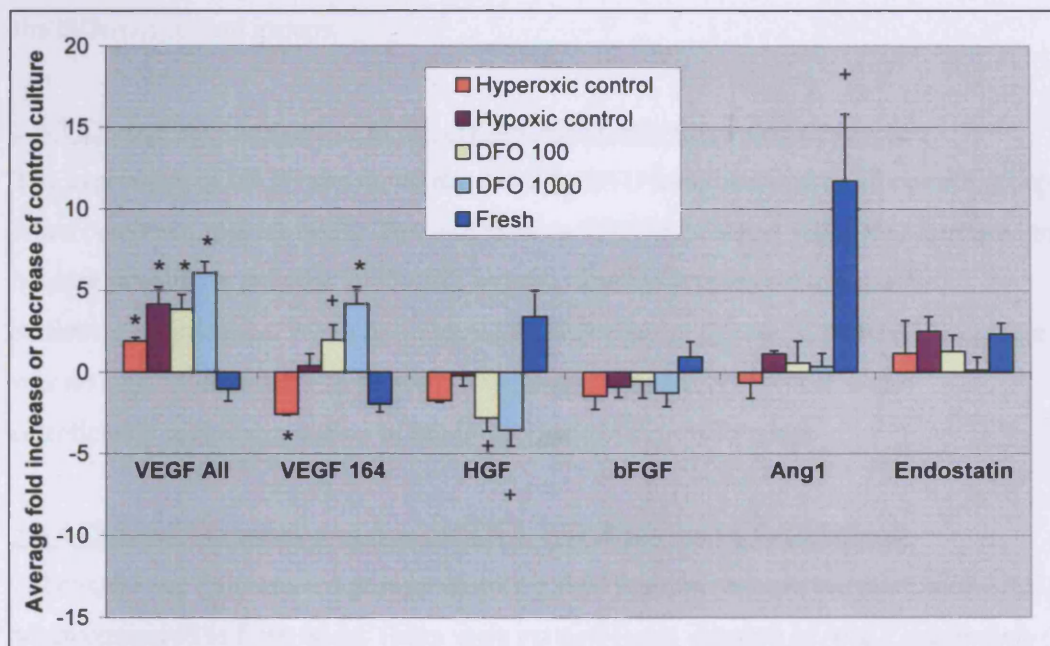


Figure 5.5: The effect of desferrioxamine treatment on the expression of angiogenic growth factor RNA expression, compared to control cultured islets. The values are presented as average fold change plus standard error. Statistical significance: p value of less than 0.01 is indicated by * and a value of less than 0.05 is indicated by +, when compared to control cultures.



5.3.1 Change in expression of VEGF mRNA with desferrioxamine treatment

Desferrioxamine treatment produced a significant, dose responsive increase in the expression of all isoforms of VEGF and VEGF164, when compared to both fresh and control cultured islets. The increase in all isoforms of VEGF with DFO treatment was similar to that induced by hypoxic culture but DFO treatment increased VEGF164 expression to a greater extent than hypoxic culture, 2.37 fold ($p=0.04$) DFO 100 and 4.14 fold ($p=0.001$) increase in VEGF164 when compared to hypoxic controls. Hyperoxic culture significantly increased all VEGF isoform expression but suppressed VEGF 164 expression when compared to fresh and control cultured islets.

5.3.2 Change in expression of HGF mRNA with desferrioxamine treatment

Hepatocyte growth factor expression was significantly down regulated by DFO treatment when compared to both fresh and control cultured islets. The pattern was related to concentration of DFO with 1000 μ M producing greater suppression than the 100 μ M treatment. All controls produced a non significant reduction in HGF expression when compared to fresh islets, with no difference in the extent of suppression between the different control groups.

5.3.3 Change in expression of bFGF mRNA with desferrioxamine treatment

The expression of bFGF was down regulated by DFO treatment and in all control groups, when compared to fresh islets. The reduction in bFGF expression with DFO appeared to be dose related but only the DFO1000, hypoxic and hyperoxic controls reached statistical significance. When the changes were compared to control culture islets, there was no significant difference between any groups. The fresh islets had a non significantly higher expression of bFGF compared to cultured islets.

5.3.4 Change in expression of Ang1 mRNA with desferrioxamine treatment.

All control and DFO treated groups showed a significantly reduced expression of Ang1, when compared to fresh islets. There were no significant changes in Ang 1 expression compared to control cultured islets, except for the fresh islet group.

5.3.5 Change in expression of endostatin mRNA with desferrioxamine treatment

There were no significant changes in the expression of endostatin when compared to either fresh or control islets. In addition, there was no obvious pattern to the changes in expression.

5.4 Effect of desferrioxamine treatment on islet viability

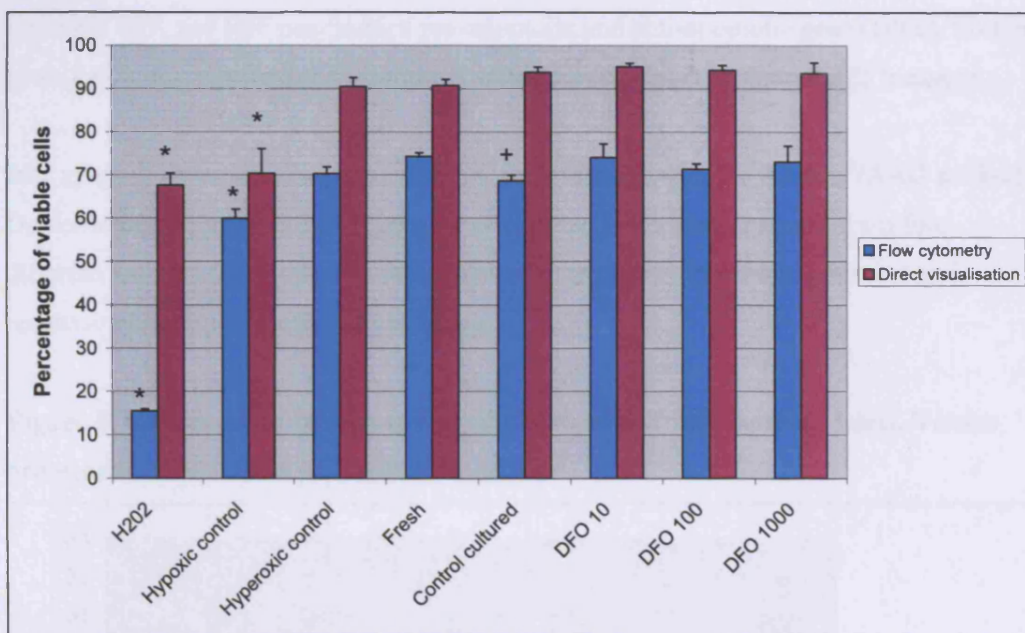
The initial experiments had shown that desferrioxamine treatment altered the expression of a number of growth factors and appeared to be a promising technique for up regulating the expression of VEGF. In addition to HPH, haem compounds and other important cellular enzymes require iron and depletion of iron from cells can lead to arrest of cell cycling through the inhibition of ribonucleotide reductase (Cooper, Lynagh et al. 1996). Therefore, it was important to ascertain that desferrioxamine treatment did not affect the viability of islets.

The viability of DFO treated and control islets was assessed by direct visualisation and flow cytometry, figure 5.6. The two different methods were used to assess any adverse effect of disrupting the islets before flow cytometry evaluation. Four sets of islets were assessed within each group. The flow cytometry measurements were analysed in triplicate and the direct visualisation was carried out on four sets of islets within a sample.

When analysed by direct visualisation, the viability of cells within islets was not significantly different in the DFO treated (95.1%- DFO10, 93.5%- DFO100, 94.3%- DFO 1000), control cultured (93.7%) or hyperoxic control islets (90.5%) when compared to fresh islets (90.7%) but viability was significantly lower in the hypoxic control (70.3%, $p=0.001$) and hydrogen peroxide treated (67.6, $p<0.001$) islets (figure 5.6). This pattern of viability was similar when analysed by flow cytometry, although the viability in the control culture group was also significantly lower than fresh islets: Fresh 74.3%, hyperoxic control- 70.3%, DFO10- 74.0%, DFO100- 71.3%, DFO1000- 73.0%, control culture- 68.6% ($p=0.011$), hypoxic control- 59.9% ($p=0.001$), hydrogen peroxide treated-15.5% ($p<0.001$).

Within each group, the viability was significantly lower when analysed by the flow cytometry method compared to direct visualisation. This effect may be due to a loss of viability during the disruption process.

Figure 5.6: Viability of cells within islets assessed by direct visualisation or dispersion and flow cytometry. Islets treated with hydrogen peroxide acted as positive control. Other groups were hypoxic controls, hyperoxic controls, freshly isolated, control cultured or desferrioxamine treated with DFO. Data are presented as mean percentage plus standard error. Statistical significance: p value of less than 0.01 is indicated by * and a value of less than 0.05 is indicated by +, when compared to fresh islets.

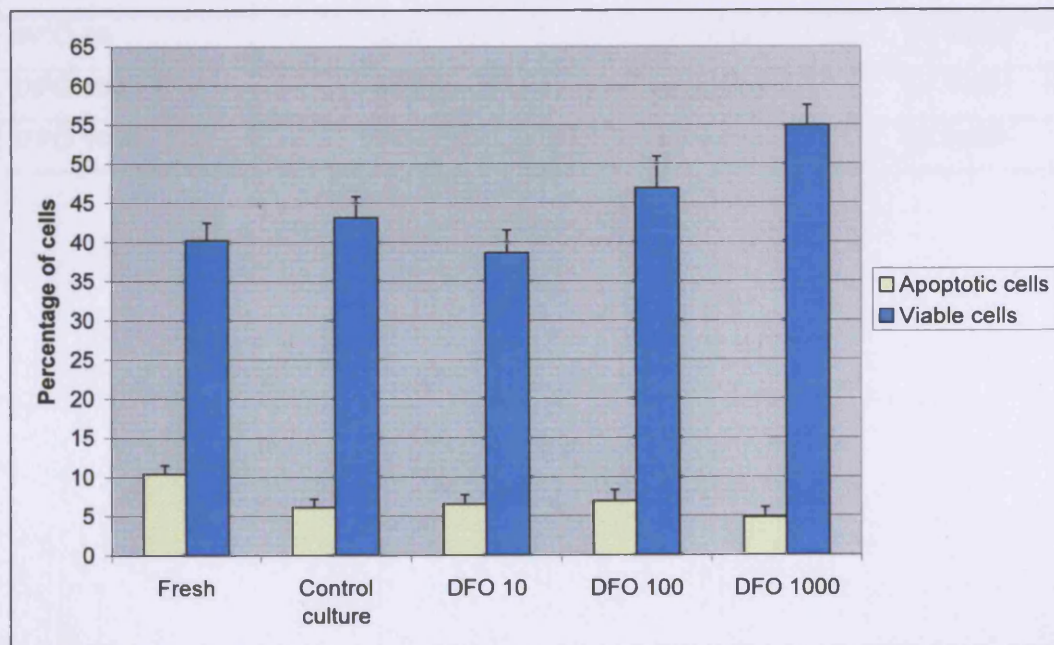


5.5 Effect of desferrioxamine treatment on islet apoptosis

The islet viability experiments did not suggest any immediate and irreversible adverse effect of desferrioxamine treatment on the viability of islets. Unfortunately, viability is a crude test and non viable cells may be the endpoint of a more subtle process, such as apoptosis. If the desferrioxamine treatment of islets induced apoptosis, it is possible that this would not be appreciated by assessing viability and islets with early apoptosis would be transplanted, leading to subsequent loss. Given that desferrioxamine treatment up-regulates HIF, and HIF may induce pro-apoptotic and anti-apoptotic genes (Piret, Mottet et al. 2002), the assessment of apoptosis would be an important endpoint to measure.

Islet apoptosis was therefore assessed in dispersed islets with the Annexin/7AAD method. Desferrioxamine treated, fresh and control cultured islets were assessed from four different animals. The assays were performed in triplicate for each experiment and results are expressed graphically in figure 5.7.

Figure 5.7: Assessment of apoptotic and viable cells from dispersed islets. Values are expressed as means plus standard error.



The level of apoptosis in the control cultured and desferrioxamine treated islets was significantly lower than in the fresh islets (control fresh- 10.4%, control cultured- 6.1% ($p<0.001$), DFO10- 6.5% ($p<0.001$), DFO 100- 6.9% ($p<0.001$), DFO 1000- 4.8% ($p<0.001$)). There was no significant difference in the percentage apoptosis between the control cultured and desferrioxamine treated groups.

The level of viable cells was much lower when assessed by 7AAD than when assessed by PI, as above. In addition, there was a difference in the percentage of viable cells between the different groups, with significantly better viability in the DFO 1000 group (table 5.1)

Table 5.1: Percentage viable cells in control and desferrioxamine treated groups when assessed by 7AAD in dispersed islets.

	Percentage of viable cells		Significance cf control fresh	Significance cf control culture
Control fresh	40.2		-	-
Control culture	43.1		$p= 0.185$	-
DFO 10	38.6		$p= 0.505$	$p= 0.068$
DFO 100	46.9		$p= 0.035$	$p= 0.193$
DFO 1000	54.9		$p< 0.001$	$p< 0.001$

5.6 Effect of desferrioxamine treatment on glucose stimulated insulin release

The experiments so far did not shown any adverse effect of desferrioxamine treatment on islet integrity. In view of the ability of HIF-1 to affect genes necessary for glucose uptake and metabolism (Wenger 2000), the metabolic response of DFO treated islets to glucose was explored. The glucose related insulin release and insulin content of control and desferrioxamine treated islets was assessed by glucose stimulation tests and measurement of islet insulin content.

5.6.1 Glucose stimulation tests

Glucose stimulation tests (figure 5.8) in each group were performed on eleven sets of islets isolated from Lewis rats (~310g).

The glucose stimulated insulin release was not adversely affected by desferrioxamine treatment but conversely, DFO 1000 islets had a significantly higher stimulation index than control fresh or control cultured islets ($p=0.024$ and $p=0.020$ respectively), table 5.2.

Figure 5.8: Insulin concentrations in supernatants from glucose stimulation tests performed on control and desferrioxamine treated islets. Data are presented as mean concentration plus standard error.

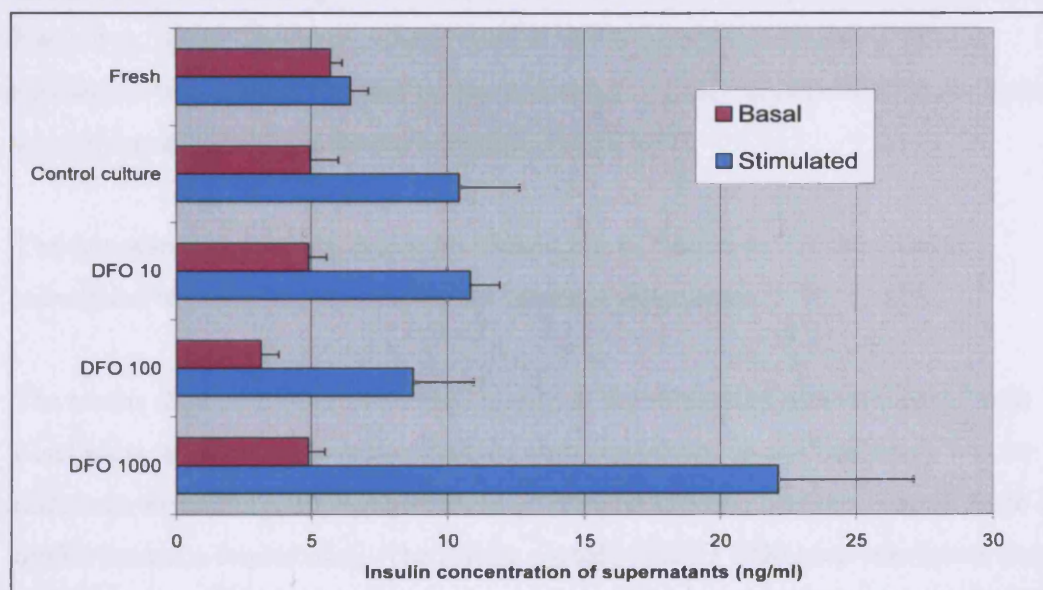


Table 5.2: Stimulation indices from glucose stimulation tests performed on control and desferrioxamine treated islets. Data are presented as mean value + 1 standard deviation.

Islets	Stimulation index
Control fresh	1.13 (+/- 0.52)
Control cultured	2.10 (+/- 1.40)
DFO 10	2.21 (+/- 0.86)
DFO 100	2.77 (+/- 2.05)
DFO 1000	4.52 (+/- 3.80)

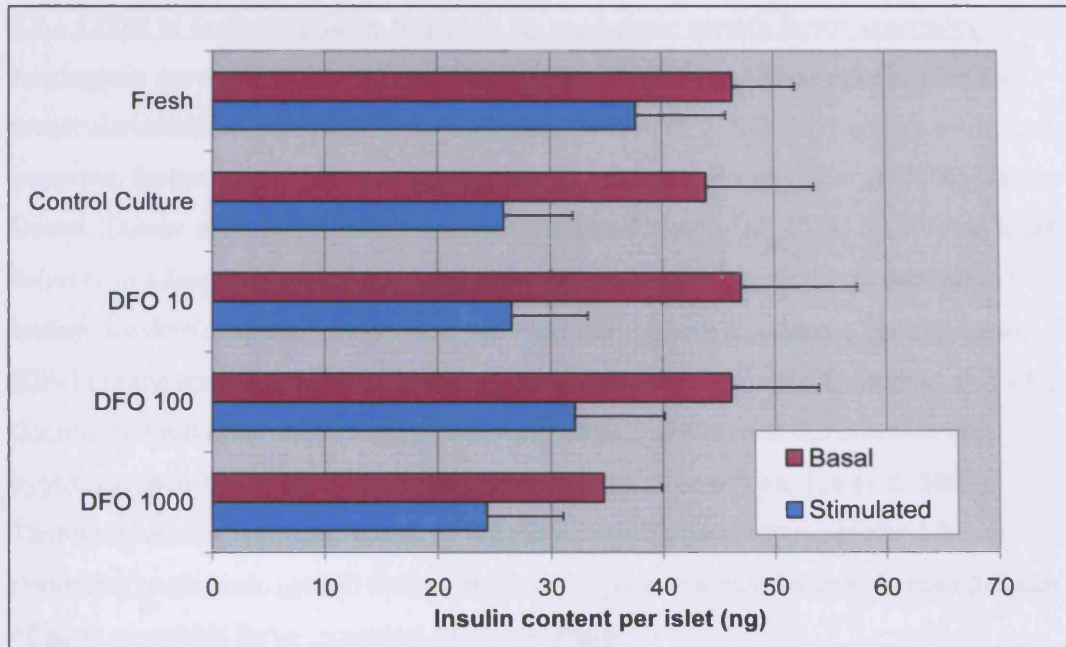
5.6.2 Insulin content of islets

The data from the glucose stimulation tests showed an improved glucose related insulin release in islets treated with higher doses of DFO which would suggest that desferrioxamine treatment may enhance the function of islets. Although glucose stimulation tests can give an indication of the functional capacity of islets, there is no correlation between stimulation indices and outcome post transplantation (Migliavacca, Nano et al. 2004). Insulin content of islets does correlate with outcome, with higher insulin content correlating with an improved outcome post transplantation (Migliavacca, Nano et al. 2004). Therefore, insulin content of the islets from the above glucose stimulation tests (figure 5.9) was assessed in order to gauge whether the insulin content was similar in control and desferrioxamine treated islets.

The data were generated by lysing the eleven sets of islets used for the glucose stimulation tests above and assaying the resultant supernatant.

The results showed a lower content of insulin in the stimulated islets compared with basal islets, although none of the changes were significant. In addition, there was no difference in insulin content, either basal or stimulated islets, between control, fresh or desferrioxamine treated islets. The insulin content of DFO 1000 islets was lower than the other groups, although not significantly so.

Figure 5.9: Mean insulin content per islet of control and desferrioxamine treated islets. Data are presented as mean concentration plus standard error.



5.7 Discussion

5.7.1 Effect of desferrioxamine treatment on angiogenic growth factor expression

Angiogenic growth factors, such as VEGF, have been shown to be important for the revascularization of transplanted islets (Vasir, Reitz et al. 2000). Up regulation of these important factors can improve islet engraftment (Nakano, Yasunami et al. 2000; Garcia-Ocana, Takane et al. 2003; Lopez-Talavera, Garcia-Ocana et al. 2004) but rely on local delivery or adenoviral techniques which would not be easy to transfer to the clinical setting. Desferrioxamine treatment of cells has been shown to increase the expression of HIF-1 (Wang and Semenza 1993; Wang, Jiang et al. 1995; Cooper, Lynagh et al. 1996; Gleadle and Ratcliffe 1997; Linden, Katschinski et al. 2003) and the down-stream hypoxia response elements that lead to angiogenesis (Yamakawa, Liu et al. 2003). Therefore, desferrioxamine treatment presents a novel and attractive method for up regulating angiogenic growth factors in islets that does not require genetic manipulation of islets or growth factor treatment of the recipient.

5.7.1a Expression of vascular endothelial growth factor

Vascular endothelial growth factor, or vascular permeability factor as it was originally known, was first isolated in 1983 (Senger, Galli et al. 1983) and belongs to a family of growth factors which include VEGF A-D and placental growth factor (Neufeld, Cohen et al. 1999). From this family of growth factors, VEGF A is the most studied and is commonly referred to as just VEGF. Therefore, all citations relating to vascular endothelial growth factor in this thesis will refer to VEGF A.

Vascular endothelial growth factor is a powerful endothelial mitogen which exerts its action through two endothelial receptor tyrosine kinases, VEGFR-1 (or Flt-1) and VEGF-2 (KDR) (Ferrara 2004). The main action of VEGF is to induce the growth of vascular endothelial cells and promote their survival but VEGF can also encourage vascular permeability (Dvorak, Brown et al. 1995) and may have some haemopoietic effects (Broxmeyer, Cooper et al. 1995).

The human VEGF gene is encoded by 8 exons (Tischer, Mitchell et al. 1991) which are alternatively spliced to form five different isoforms that are denoted by their amino acid length (VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆). The rat gene similarly has 8 exons (Tober, Cannon et al. 1998) which results in a 90% amino acid homology to the human VEGFs but the rat VEGFs lack an amino acid at the N terminal portion (Breier, Albrecht et al. 1992), therefore leading to isoforms VEGF₁₂₀, VEGF₁₄₄, VEGF₁₆₄, VEGF₁₈₈ and VEGF₂₀₅. The VEGF₁₆₄ isoform is the most predominant in rats (Bacic, Edwards et al. 1995) and is increased in the early stages of islet engraftment (Vasir, Jonas et al. 2001). Further to this observation, a number of authors have up regulated the expression of VEGF₁₆₄ in transplanted islets and have reported a variety of improved outcomes (Zhang, Richter et al. 2004; Chae, Lee et al. 2005; Lai, Schneider et al. 2005). Therefore, the effect of DFO on the expression of VEGF was explored.

Desferrioxamine up regulated the expression of VEGF protein in a predictable dose dependent manner. In addition, the higher doses of desferrioxamine (DFO100μM and DFO1000μM) induced a sustained increase in VEGF protein production after transfer to an iron-containing medium, suggesting that VEGF over expression may continue after transplantation into the iron rich environment of the portal vein. This pattern of expression is unlikely to be due to low iron availability within the culture medium as M-199 contains free iron and the medium was supplemented with FCS, which contains free and bound iron. The explanation for the sustained VEGF production is most likely to be due to the hydrophilic property of DFO reducing its exit from cells (Richardson, Ponka et al. 1994; Cooper, Lynagh et al. 1996) and prolonging the chelation of labile iron pools. This property of DFO provides a unique method for prolonging the increased expression of VEGF after transplantation that will eventually wash out after a few days. Conversely, the hydrophilic property that causes retention of DFO within cells also requires time for the ingress of DFO into cells (Cooper, Lynagh et al. 1996). Therefore, islets would need to be exposed to higher concentrations of DFO (100μM or more) for at least 4 hours in culture (Cooper, Lynagh et al. 1996).

The increase in VEGF protein expression with DFO was mirrored by the changes in the VEGF mRNA expression, with a dose dependent increase in both all isoforms of VEGF and VEGF₁₆₄. The benefit of analysing mRNA expression is the comparison with different controls, such as fresh islets, which would not be possible with protein expression. Although the changes with DFO were predictable, the changes with hypoxia and hyperoxia require further explanation.

Hypoxia increased the expression of all isoforms of VEGF to a similar extent as treatment with DFO 100 μ M but the VEGF₁₆₄ isoform was up regulated to a lesser degree and was not significantly higher than in the control cultured islets. This result was reinforced by the VEGF protein data and reflects the fact that the R&D systems ELISA is specific for the 164 and 120 isoforms. Differential expression of the VEGF isoforms in response to oxygen tensions has been reported before and in an in-vivo rat pup model of retinal vascularisation, the VEGF₁₂₀ and VEGF₁₈₀ isoforms were up regulated to a greater extent than VEGF₁₆₄ in response to a hypoxic stimulus (McColm, Geisen et al. 2004). The study also showed a greater reduction in the expression of VEGF₁₆₄ to hyperoxia (50% O₂) than the VEGF₁₂₀ and VEGF₁₈₀ isoforms (McColm, Geisen et al. 2004). This observation is mirrored in these results where VEGF₁₆₄ expression was reduced by hyperoxia but other isoforms were not. The reason for this difference in isoform expression is not clear but may be because VEGF is not only regulated by transcriptional induction (Neufeld, Cohen et al. 1999; Ferrara 2004) but its expression can be induced by changes in mRNA stability (Levy, Levy et al. 1995; Levy, Levy et al. 1996). Therefore, the differences in mRNA structure between isoforms may account for differences in stability or induction under different conditions.

Despite the differences between VEGF isoforms, the change in VEGF₁₆₄ expression induced by hypoxia (exposure to 1% oxygen mix) was less than expected. The cause of this poor up regulation is probably multifactorial. Firstly, the hypoxic stimulus of 1% ambient oxygen (7.5mmHg) may not have been sufficient to maximally induce VEGF₁₆₄. The in-vitro 50% maximal induction of VEGF₁₆₅ in human cervical cancer cell lines has been estimated to be between 13 and 27mmHg, depending on the cell line, with maximal

induction occurring at 0 to 6.3 mmHg (Chiarotto and Hill 1999). Our model did not directly measure the partial pressure of oxygen within media and despite the 20 minute gassing of modular chambers, it is possible that either the gas did not fully equalize or there was additional oxygen dissolved within the media. For this reason, it may have been better to use a lower oxygen concentration for induction of VEGF, such as 0.5%, 0.1% or 0% oxygen, as described by other authors (Pham, Uchida et al. 2002; Turner, Crew et al. 2002; Nilsson, Shibuya et al. 2004) or a longer incubation time. However, this was not the experience of Vasir et al. who showed a 3.8 and 2.6 fold induction of VEGF₁₆₄ in rat islets exposed to 24 hours of 1% oxygen, when compared to fresh and normoxic cultured islets respectively (Vasir, Aiello et al. 1998). In addition, the culture of islets exposed to 1% oxygen has been shown to up regulate HIF-1 α protein expression after only 1 hour, which suggests that 1% ambient oxygen should be a sufficient stimulus to the induction of hypoxia response elements such as VEGF (Moritz, Meier et al. 2002).

Secondly, the fold change in mRNA expression is a relative change and fresh and control cultured islets were used as the reference for gene expression. Both of these reference points require isolation and manipulation of islets which may affect the expression of growth factor mRNA. In cultured cells, the effect of stirring (Chiarotto and Hill 1999) or changing cellular architecture (Milsom and Rak 2005) can increase the expression of VEGF and other angiogenic growth factors. Therefore, the manipulation required to isolate islets may have altered baseline gene expression in control islets and reduced the effect of increased expression in the experimental islets. A better control may have been fresh whole pancreas, although this control may influence gene expression patterns as the sample would contain exocrine tissue.

5.7.1b Expression of Hepatocyte growth factor

Hepatocyte growth factor, or scatter factor, is a heterodimeric molecule consisting of an alpha and beta subunit (Funakoshi and Nakamura 2003). HGF is secreted as an inactive form but is activated by serine proteases and exerts its actions through the c-MET receptor (Funakoshi and Nakamura 2003). The active molecule is a potent mitogen to primary cultured hepatocytes and has an important role in organogenesis of the liver and other organs, with embryonic lethality in knockout animals (Uehara, Minowa et al. 1995; Uehara, Mori et al. 2000). Hepatocyte growth factor has also been shown to reduce endothelial cell death due to hypoxia (Hayashi, Morishita et al. 1999), protect from ischaemia induced apoptosis (Funakoshi and Nakamura 2003) and promote angiogenesis (Hayashi, Morishita et al. 1999; Taniyama, Morishita et al. 2001).

The pro angiogenic and anti-apoptotic properties have made HGF an attractive target for improving the survival and revascularization of transplanted islets and a number of authors have looked at this issue by either adenoviral transfection (Garcia-Ocana, Takane et al. 2003; Lopez-Talavera, Garcia-Ocana et al. 2004) or increased local delivery techniques (Nakano, Yasunami et al. 2000). The increased expression of HGF either locally or in transplanted islets has been shown to improve glucose normalisation and histological endpoints in marginal islet transplant models (Nakano, Yasunami et al. 2000; Garcia-Ocana, Takane et al. 2003; Lopez-Talavera, Garcia-Ocana et al. 2004). In view of these studies and the fact that co administration of HGF with VEGF enhances angiogenesis in-vivo and vitro over the single molecules alone (Xin, Yang et al. 2001), it was hoped that DFO would also up regulate HGF.

Hepatocyte growth factor was significantly and dose dependently down regulated by DFO. This finding was further supported by a four fold trend toward reduced expression of HGF in response to hypoxia. Although HGF does have angiogenic effects, its expression has been shown to be down regulated by hypoxia both in-vitro (Hayashi, Morishita et al. 1999; Corpechot, Barbu et al. 2002) and in-vivo (Morishita, Nakamura et al. 1999).

The expression of HGF was also down regulated, although not significantly, in control cultured and hyperoxic treated islets when compared to fresh islets. This suggests that hypoxia is not the only determinant of HGF expression and that the culture of islets may have a negative impact on HGF expression. If HGF is truly necessary for islet engraftment (Nakano, Yasunami et al. 2000), the impact of culture on its expression may explain the impaired vascular density and normalisation of hyperglycaemia post transplant of cultured islets when compared to fresh islets (Olsson and Carlsson 2005).

5.7.1c Expression of basic fibroblast growth factor

The fibroblast growth factors consists of 22 members, of which basic fibroblast growth factor, also known as FGF-2, is a single member (Ornitz and Itoh 2001). The basic fibroblast growth factor gene extends over 36kb and consists of 3 exons with large 3' and 5' untranslated regions (UTR), suggesting its regulation may be transcriptional (Okada-Ban, Thierry et al. 2000). The transcription of the gene is bidirectional, giving rise to a 1.5kb antisense transcript complementary to the 3' UTR which has been implicated in the transcriptional and post transcriptional regulation of bFGF expression (Knee and Murphy 1997).

The translation of the gene is further complicated by multiple non-classical CUG start codons 5' to the classical AUG start codon which results in 5 protein isoforms of 18, 22, 22.5, 24 and 34kDa (Florkiewicz and Sommer 1989; Prats, Kaghad et al. 1989; Delrieu 2000). The classical AUG start codon gives rise to the smaller 18kDa isoform and translation starting at the other non classical CUG start codons leads to formation of the larger molecular weight isoforms. The expression of the different bFGF isoforms is complex and although the 18kDa isoform may be up regulated at the transcriptional level, the larger CUG initiated isoforms are differentially regulated at the translational level, independent of mRNA expression (Vagner, Touriol et al. 1996). This has been shown in an in-vitro model where oxidative stress and heat shock up regulated the large molecular weight isoforms but not the 18kDa isoform (Vagner, Touriol et al. 1996).

The small 18kDa isoform is mainly cytosolic but the larger isoforms contain a nuclear localising sequence which directs the protein to the nucleus (Okada-Ban, Thiery et al. 2000). The larger isoforms tend to have nuclear targets but the 18kDa isoform can be secreted into the extracellular medium and may have autocrine or paracrine effects through FGF receptors (Okada-Ban, Thiery et al. 2000). The smaller isoform was initially shown to be mitogenic to fibroblast cells but other actions include endothelial morphogenesis and proliferation leading to angiogenesis, and protection of neural cells from apoptosis (Okada-Ban, Thiery et al. 2000). The angiogenic properties of bFGF have interested researchers in the field of islet revascularisation and early studies suggested that a local increase of bFGF protein could enhance normalisation of glycaemia (Hayek, Lopez et al. 1990) and improve vascular density within transplanted islets (Hayek, Lopez et al. 1990). In addition, bFGF has been shown to augment the vascular smooth muscle (Schultz, Fanburg et al. 2006) and endothelial cell proliferation (Calvani, Rapisarda et al. 2005) in response to HIF and hypoxia. Therefore, it was hypothesised that DFO might up regulate both bFGF and HIF-1.

The investigation of bFGF in islets produced different results depending on whether mRNA or protein expression was analysed. The mRNA results suggested that cultured islets had a lower expression of bFGF compared to fresh islets but there was no difference in mRNA expression between any of the groups when control cultured islets were used as the reference point. When bFGF protein was analysed, there was no significant difference between any of the groups except for the hypoxic islets, where bFGF protein was significantly increased. Therefore, hypoxic treatment of islets increases bFGF protein but not mRNA expression while DFO treatment does not affect either bFGF protein or mRNA levels. Although the specificity of the ELISA for bFGF isoforms is not listed, I have assumed that the ELISA measures all protein isoforms. If so, these results would suggest that hypoxia induces bFGF protein expression by translational modification that is independent of hypoxia inducible factor. A mechanism for this pattern of expression could be hypothesised through the complex transcriptional/translational regulation of bFGF expression. Other authors have also shown a similar response of protein up regulation without changes in mRNA expression

in response to hypoxia (Ishibashi, Shiratuchi et al. 2001). A further study by Brogi et al. using a human vascular smooth muscle cell culture model found that hypoxia up regulated VEGF mRNA expression but not bFGF mRNA expression and also postulated that HIF-1 may have no effect on bFGF mRNA expression (Brogi, Wu et al. 1994). Contrary to this finding, a further study using human umbilical vein endothelial cells has shown that 1% oxygen up regulates both bFGF protein and mRNA expression which could be blocked by the use of HIF1- α siRNA, therefore suggesting that both protein and message are regulated by hypoxia inducible factor (Calvani, Rapisarda et al. 2005). Therefore, it remains to be determined whether these results are reproducible and whether HIF-1 controls bFGF expression.

These results suggest that neither culture nor DFO treatment significantly affect bFGF expression although the trend was toward a reduction in expression when compared to fresh islets. Once again, this suggests a further hypothesis as to why cultured islets seem to do worse than fresh after transplantation.

5.7.1d Expression of angiopoietin 1

The angiopoietins are unusual angiogenic factors which contain three separate domains. The first is an N terminal section which has no known structural homology. The N terminal domain is followed by a coiled-coil domain and a final C terminal domain that has close homology to fibrinogen (Davis, Aldrich et al. 1996). The angiopoietin family consists of four members Ang1-4, of which Ang-3 is found only in mice and Ang-4 is its human counterpart (Valenzuela, Griffiths et al. 1999). Ang-1 is an agonist for the Tie-2 receptor and, unlike a number of angiogenic factors, was discovered after its receptor (Davis, Aldrich et al. 1996). Ang-1 appears to orchestrate the normal interaction between endothelial cells and supporting cells which leads to improved vascular stability (Thurston, Rudge et al. 2000; Loughna and Sato 2001). This property of Ang-1 was highlighted by a study that used a transgenic mouse which over expressed Ang-1. The over expression of Ang-1 in a model of increased VEGF expression caused reduced capillary leakage induced by the over expression of VEGF. Ang-1 is not a strong endothelial mitogen (Davis, Aldrich et al. 1996) but probably works in conjunction with

VEGF to promote vascular remodeling and endothelial sprouting (Maisonpierre, Suri et al. 1997). Ang-2 is a natural antagonist of the Tie-2 receptor and probably works by balancing the effect of Ang-1.

The Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) receptors were found to be predominantly endothelial based. Their function was initially investigated using knock out mice and Tie 1 null mice embryos have subcutaneous oedema and hemorrhaging leading to late embryonic death (Sato, Tozawa et al. 1995), suggesting that Tie-1 is important for vascular integrity. The Tie-2 null mice had extensive malformations of early vasculature with early embryonic death (Sato, Tozawa et al. 1995) which suggests that Tie-2 is involved in network formation and maintenance during angiogenesis.

Increased VEGF expression has been shown to improve outcomes after transplantation but there was no evidence about the effect of VEGF up regulation on vascular integrity or supporting cells within transplanted islets. Given that Ang-1 can improve the poor vascular integrity associated with VEGF up regulation (Thurston, Suri et al. 1999), I was interested to see whether DFO would be able to regulate both angiogenic molecules together.

All the cultured groups showed a significant down regulation of Ang-1 when compared to fresh islets, with no difference between DFO treated, hypoxic or hyperoxic controls when compared to control cultured. While this result was initially disappointing, it is possible to postulate the mechanism for the reduced expression. Our in-vivo data had already shown regression of endothelium after the first day of transplantation and the reduced Ang-1 expression in cultured islets would be consistent with endothelial dissolution. In addition, hypoxia and HIF-1 are known to up regulate the expression of Ang-2 and Ang-4 while inducing tube formation of endothelium (Yamakawa, Liu et al. 2003). Hypoxia in-vivo has also been shown to down regulate Tie-2 and Ang-1 mRNA expression in a variety of tissues (Abdulmalek, Ashur et al. 2001). This balance would suggest that in the early stages of angiogenesis, Ang-1 and the effect of Tie-2 is

probably down regulated in order to increase the flexibility of new vessel formation. Therefore, it may be more informative to look at levels of Ang-2 which have been shown to be increased with VEGF during angiogenesis and may potentiate VEGF-induced neovascularisation (Hackett, Ozaki et al. 2000).

The expression of Ang-1 during islet revascularization has not been elucidated so far but our results suggest that culture reduces the expression of Ang-1 when compared to fresh islets. In view of the necessity of Tie-2 action for coordinated vasculogenesis (Sato, Tozawa et al. 1995) and Ang-1 expression for developing leakage resistant vessels induced by VEGF (Thurston, Suri et al. 1999), the down regulation of Ang-1 during culture may be an additional reason for poor revascularization of cultured islets (Olsson and Carlsson 2005).

5.7.1e Expression of endostatin

Endostatin is a compact globular protein that is proteolytically cleaved from the C terminal end of collagen XVIII (Sim, MacDonald et al. 2000; Abdollahi, Hlatky et al. 2005). Endostatin has been shown to reduce expression of VEGF (Hajitou, Grignet et al. 2002), inhibit VEGF induced tubularisation of endothelium (Ergun, Kilic et al. 2001) and promote endothelial apoptosis (Dhanabal, Ramchandran et al. 1999). The anti-angiogenic effect of endostatin has been investigated in a mouse tumour model where exogenous endostatin reduced the vascularity and size of experimentally induced haemangioblastomas (O'Reilly, Boehm et al. 1997).

The mechanism for regulation of endostatin is unclear and although it has been suggested that endostatin down regulation does not induce angiogenesis by changes in HIF-1 (Macpherson, Ng et al. 2003), it is not known whether HIF-1 expression alters endostatin expression. Hypoxia has been reported to down regulate endostatin protein expression in human endothelial cells, pericytes and endometrial cells (Wu, Yonekura et al. 2001; Nasu, Nishida et al. 2004) but chronic hypoxia has been shown to up regulate endostatin protein in a number of murine tissues (Paddenberger, Faulhammer et al. 2006). I was unable to show a change in endostatin mRNA expression in response to hypoxia,

hyperoxia or DFO treatment. This lack of mRNA expression change with hypoxia has been reported before and when combined with changes in endostatin protein, has lead the authors to conclude that endostatin regulation is post translational (Wu, Yonekura et al. 2001; Nasu, Nishida et al. 2004). Further experiments could therefore examine protein expression of endostatin rather than mRNA levels.

5.7.1f Angiogenic factor expression and effect

These experiments have shown that DFO can alter the expression of angiogenic factors in islets in a similar but with more exaggerated pattern than moderate hypoxia. This pattern of expression provides a useful insight into the effect of DFO on islets but does not provide the whole picture of its end organ effects. A number of the angiogenic growth factor receptors are also modulated by hypoxia (Neufeld, Cohen et al. 1999; Vasir, Reitz et al. 2000; Yamakawa, Liu et al. 2003) which leads to a change in the end organ effect of the angiogenic factor. I have not examined the effect of DFO and culture on receptor expression but this would be an important endpoint for evaluating the full effect of these treatments.

Isolated islets produce growth factors in culture and in response to hypoxic signal. These experiments have examined the expression of growth factor protein and mRNA but have not looked at where the growth factors originate from or the change in their time course after transplantation. Although the growth factor sequences were obtained, amplified and sequenced using IMAGE clones, I was unable to develop the in-situ hybridization technique for analysing RNA expression in tissue sections. The analysis of isolated and transplanted islets will provide further data on the origin of growth factor expression and, although not quantifiable, could provide insight into the post transplant time course of their expression.

5.7.2 Effect of desferrioxamine treatment on islet viability

Iron is an important cofactor in a number of cellular enzymes and the effects of desferrioxamine treatment may affect cellular functioning and viability. In addition, HIF-1 may exert both pro-apoptotic and anti-apoptotic effects with the apoptotic effects becoming more evident after prolonged hypoxia (Piret, Mottet et al. 2002). It is possible that the effects of desferrioxamine treatment and HIF-1 over-expression may have had negative effects on the islets by either inhibiting proliferation or increasing apoptosis in the grafts.

The viability assessment techniques deployed here worked well with good correlation between both methods. Despite the good correlation, the viability assessed by flow cytometry was numerically lower than the direct visualisation group. This observation has been recorded by other authors using flow cytometry (73.2% viability) versus confocal microscopy (86.3% viability) (Boffa, Waka et al. 2005) and is probably due to the disruption of islets prior to assessment. Despite the use of a non trypsin based technique, the disassociation of adherent cells probably leads to the reduced viability seen between these techniques but when each individual technique was used (flow cytometry or direct visualisation), the results allowed comparison between different experimental groups.

The viability assessment of iron depleted islets showed that DFO treatment did not significantly reduce viability when compared to fresh islets. When the viability was compared to the hypoxic control islets, there was a significantly higher viability in the both DFO treated groups using either technique. This pattern of viability suggests that although DFO can mimic the growth factor profile of hypoxia, it does not have the associated reduction in viability. Although the viability within islets in the control cultured group was lower than in fresh islets, when assessed by flow cytometry, there was no significant difference between control cultured and DFO treated groups. Therefore, it is impossible to say whether DFO may have protected against any loss of viability in culture.

If DFO does have a hypoxia preconditioning effect, further experiments should assess the viability of control cultured islets and DFO treated islets after both groups had been exposed to a further 24 hours of hypoxic culture. This experimental design would simulate control cultured and DFO treated islets being transplanted into the hypoxic environment of the liver and may help examine whether DFO could hypoxia precondition islets prior to transplantation.

5.7.3 Effect of desferrioxamine treatment on islet apoptosis

The islet viability experiments suggested that desferrioxamine treatment did not have a negative impact on the early survival of cells within islets but it is not clear whether desferrioxamine treatment may initiate apoptosis. The increased expression of HIF-1 α in islets exposed to hypoxic culture conditions has been shown to induce apoptosis (Moritz, Meier et al. 2002) and it would be important to examine whether the induction of HIF-1 by treatment with DFO had the same affect. The initiation of apoptosis would not be revealed by the use of cell exclusion dyes and cells within islets may then progress to late apoptosis at the time of transplantation, with subsequent loss of graft mass.

The assessment of apoptosis presented here suggests that desferrioxamine treatment has no affect on islet apoptosis but the results have to be interpreted in light of the technique used. Our modification of the Cattani technique (Cattani, Berney et al. 2001) still required the use of islet disruption which may have increased the rate of islet apoptosis or necrosis within islets. This hypothesis is further backed up by the difference in cell viability between disrupted and non disrupted islets (in above section) and suggests that disruption and flow cytometry can negatively affect viability. Despite the need for disruption, the assessment presented here suggested lower values of apoptosis when compared to the description of the original technique (Cattani, Berney et al. 2001) but should be interpreted in light of value presented by other authors. The values generated for annexin positive cells presented here are similar to the values presented by Boffa et al (6.4%) in their assessment of apoptosis in islets (Boffa, Waka et al. 2005). Unfortunately, their assessment of apoptosis was different and it is unclear why the authors have selected annexin positive/propidium iodide negative incorporated with

annexin positive/propidium iodide positive cells for the assessment of apoptosis because the annexin positive/propidium iodide positive cells should denote necrosis, as outlined in the original description of the technique (Vermes, Haanen et al. 1995). The values for apoptosis in islets presented by Boffa and here differ greatly from the original description of the annexin V and propidium iodide technique (21.1%) by Cattani (Cattani, Berney et al. 2001), the 7AAD and TMRE technique (40-50% of viable cells) presented by the same group in Miami (Ichii, Inverardi et al. 2005) or the TUNEL technique (1.5%) presented in Cattani's paper (Cattani, Berney et al. 2001). A further paper using acridine orange and direct visualisation suggested that islet apoptosis is closer to 1% (Hadjivassiliou, Green et al. 1998). This immense variability highlights the difficulty of assessing apoptosis in islets and suggests that once a technique is established, the relative differences between treatment groups are more important than the absolute values.

Despite the inadequacies of this technique, the results suggested that DFO treatment did not increase the rate of apoptosis within cultured islets. Conversely, the results did not suggest a reduction of apoptosis either. Apoptosis was higher in the fresh islets (10.4%) compared with the cultured islets (~6%) and this may be a result of apoptosis induced by islet isolation. Conversely, the result could represent selection bias where healthy (non apoptotic) islets may have been hand picked from culture dishes after overnight culture compared with all fresh islets being non selectively used for analysis. A further analysis using hypoxic cultured islets would have been a useful positive control for this assay, as culture of islets exposed to 1% ambient oxygen has been shown to induce apoptosis (Moritz, Meier et al. 2002).

The percentage of necrotic cells using the 7AAD/Annexin technique were much higher than when PI alone was used and are higher than previously reported (Cattani, Berney et al. 2001; Boffa, Waka et al. 2005; Ichii, Inverardi et al. 2005). The reason for this was unclear and was originally thought to be due to differences between 7AAD and PI but 7AAD has been used before on islets without such high levels of necrosis (Ichii, Inverardi et al. 2005). The reason for the higher percentage may be due to flow

cytometry set up, overlap of signal and changes with compensation or may have been the higher concentration of 7AAD used in order to generate a clear signal.

Despite the difficulties of the technique, there does not appear to be any detrimental effect of DFO on islet apoptosis.

5.7.4 Effect of desferrioxamine treatment on insulin secretion

Viability is a useful test for assessing islets but does not correlate with post transplant outcomes (Migliavacca, Nano et al. 2004). The secretory function of islets may effect their engraftment post transplantation and any procedure which modifies or manipulates islets, in order to improve their engraftment, should not reduce their functional capacity. Insulin secretion in response to a glucose challenge (glucose stimulation tests) gives some indication of the function capacity of islets in-vitro, although the results do not necessarily correlate with clinical outcomes after transplantation (Migliavacca, Nano et al. 2004).

The glucose stimulation data suggested that desferrioxamine treatment does not impair the functional capacity of islets and, in higher doses, desferrioxamine treatment may improve the glucose-induced insulin response. The cause for this increased insulin response is unclear but may be related to an up regulation of glucose transporters by HIF-1 (Gleadle and Ratcliffe 1997) leading to an increased sensitivity of islets to glucose. This hypothesis is supported by the insulin content data which suggests that the insulin content of DFO1000 islets subjected to high levels of glucose tends to be lower than that of the other groups.

When interpreting the glucose stimulation data, the results need to be compared to ranges achieved by other authors. De Haan et al (de Haan, Faas et al. 2004) used a similar method for the assessment of glucose stimulation and found an insulin secretion of around 20-25ng/ml.10 islets; a value 4-5 fold higher than those reported here. In addition, the stimulation indices of fresh and cultured islets reported here are lower than might be expected. The cause for the reduced insulin secretion is complex but donor

weight, donor strain, culture conditions and source of protein for processing are all factors which may influence the stimulation index in rodent islets (de Haan, Faas et al. 2004). The rats chosen for the in-vitro experiments were of slightly higher body mass than the rats used for transplantation and this may have negatively impacted on their stimulation indices (Davalli, Scaglia et al. 1996; Biarnes, Montolio et al. 2002). When all the individual factors are considered, the stimulation indices presented here may be explained by the different subjects used for these experiments (de Haan, Faas et al. 2004). The important fact remains that all islets were exposed to the same experimental conditions and the values attained are reproducible. Therefore, desferrioxamine treatment does not appear to have a negative impact on the secretory function of islets in-vitro and in higher doses, may enhance their response

5.7.4 Effect of desferrioxamine treatment on islet insulin content

Dynamic islet tests, such as glucose stimulation, provide some information about islet health and function but, as mentioned above, do not correlate with clinical outcome after transplantation (Bertuzzi, Secchi et al. 2004). Insulin content of islets provide a better indicator of outcome after transplantation and were therefore performed on the in-vitro islets.

The islets exposed to higher concentrations of glucose had lower insulin content than basal islets and would be consistent with the secretion of stored insulin in response to a glucose challenge. The reduction of insulin content was lowest in the fresh islets and represents the low stimulation index seen in this group of islets. When all the groups are compared, there was no significant difference in insulin content between control and DFO treated islets, suggesting that DFO did not affect insulin content. Although the change did not quite reach statistical significance, the insulin content for stimulated and basal islets exposed to DFO1000 μ M was lower than the other groups. This observation is in agreement with a possible increased sensitivity to glucose caused by HIF-1 up regulation but may also lead to a poorer outcome post transplantation, if insulin content correlates with glucose normalisation after treatment with DFO

5.8 Conclusions

These experiments have shown that the desferrioxamine treatment of islets in-vitro can induce a similar but more exaggerated pattern of angiogenic growth factor response to that seen in hypoxia. In particular, desferrioxamine treatment can produce a prolonged over expression of VEGF and does not adversely affect islet viability or apoptosis and may improve islet insulin response to a glucose challenge.

Chapter 6 – Results:

In-vivo desferrioxamine treatment studies

6.1 Introduction

The analysis of transplanted islet revascularisation (chapter 4) showed that islet engraftment is a complex process which follows a coordinated sequence of events. The coordination of these events is probably due to the expression of local growth factors which drive angiogenesis and lead to the formation of a new vascular network (Menger, Yamauchi et al. 2001). Although a number of candidate growth factors have been suggested to control revascularisation, VEGF is probably the most important factor (Menger, Yamauchi et al. 2001) and is known to be expressed early during the revascularisation process (Vasir, Jonas et al. 2001). The manipulation of VEGF expression is therefore a prime target for improving the revascularisation of islets and its up regulation has been shown to improve the insulin responsiveness and glucose normalisation post transplant in addition to increasing insulin staining and vascular density within islet grafts (Zhang, Richter et al. 2004). Despite these positive results, VEGF may not be the entire answer to islet revascularisation and experimental islet transplantation in the presence of VEGF receptor blocking antibodies has shown that islets revascularise normally when compared to controls (Schramm, Yamauchi et al. 2003). This suggests that the manipulation of multiple factors is probably required to improve revascularisation. Therefore, increasing VEGF expression by targeting the hypoxia signalling cascade with DFO may present a method for improving the engraftment of islets post transplantation.

The in-vitro results suggested that desferrioxamine treatment of islets produced a sustained increase in VEGF expression which persisted after the return to an iron containing environment. In addition, the treatment with desferrioxamine did not affect the viability or function of islets and in higher doses, may have improved glucose stimulated insulin release. It was therefore postulated that desferrioxamine treatment

could precondition islets to over express VEGF during the peri transplant and immediate post transplant period. The increased expression of VEGF would therefore bring forward the time of peak endothelial mitogen expression to the day of transplantation, rather than at day 3 (as suggested by (Vasir, Jonas et al. 2001)), and hopefully improve transplant outcome. In addition, it is possible that the hypoxia preconditioning effect of desferrioxamine seen in neuronal cells (Prass, Ruscher et al. 2002) could be replicated in islets prior to transplantation and hence reduce hypoxic cell death after transplantation. Therefore, the next experiments were designed to determine whether the in-vitro data could be transferred to the in-vivo setting and desferrioxamine treated islets could improve the outcome of experimental islet transplantation.

6.2 Effect of desferrioxamine treatment on ultra-marginal (350 islet) syngeneic islet transplants

The initial experiments with the right branch transplant model suggested that this technique may reverse streptozotocin induced diabetes with a lower dose of transplanted islets. Therefore, the initial experiments were carried out using a single donor to recipient model with a dose of 350 islets.

6.2.1 Change in blood glucose after 350 islet transplant

Three diabetic animals were transplanted in each group (fresh islets, islets cultured overnight in CMRL alone and islets treated overnight in either DFO 100 μ M or DFO 1000 μ M). There was no significant difference in mean weight or mean blood sugar between each group at the time of transplant, table 6.1. All transplanted animals survived surgery and had a moderate fall in blood glucose and rise in body weight from pre transplant baseline. One animal in the control culture group died unexpectedly 6 weeks after transplant. The cause of death was not obvious at post-mortem examination.

There was a fall in blood glucose in all groups from pre transplant levels to the day of harvest, although this was only significant in the fresh islet group. None of the animals were rendered normoglycaemic. The fall in blood glucose did not appear to be different between the groups graphically (figure 6.1) but when the overall changes in blood glucose was analysed by repeated measures ANOVA, all the cultured groups (control and desferrioxamine treated) were significantly different from the control fresh group (table 6.2). There was no difference between any of the desferrioxamine treated or control cultured groups (table 6.2). This observation was further backed up by the area under the curve analysis for blood glucose over time between the different groups (Control fresh- 1767.0mmol/l.d, control culture- 1926.4 mmol/l.d, DFO- 100 1934.0 mmol/l.d and DFO 1000- 1905.6 mmol/l.d), which suggested a lower overall blood glucose in the control fresh group compared with the other groups.

Table 6.1: Change in body weights and blood sugar concentrations of diabetic rats on the day of transplant and day of harvest (11 weeks post transplantation) after 350 islet transplants. Data is displayed as mean \pm 1 standard error. Significance is calculated by paired t test.

	Pre transplant		Day of harvest		Significance of change between time points	
	Weight (g)	Sugar (mmol/l)	Weight (g)	Sugar (mmol/l)	Weight	Sugar
Fresh	249.3(3.8)	32.5(0.37)	298.7(2.4)	25.7(1.8)	0.003	0.021
Control Culture	254 (8.1)	28.8(4.2)	304.5(11.0)	23.0(0.1)	0.044	0.365
DFO 100	243 (9.1)	30.1(2.2)	276(10.2)	27.6(3.1)	0.073	0.541
DFO 1000	243 (17.0)	26.1(3.5)	287(22.6)	21.3(3.2)	0.017	0.366

Figure 6.1: Change in mean non-fasting blood glucose over time in diabetic recipients of 350 desferrioxamine treated islets. The islets were either transplanted: fresh, overnight culture in CMRL alone (control cultured), or desferrioxamine treated (DFO 100) and (DFO 1000). Three animals were transplanted in each group.

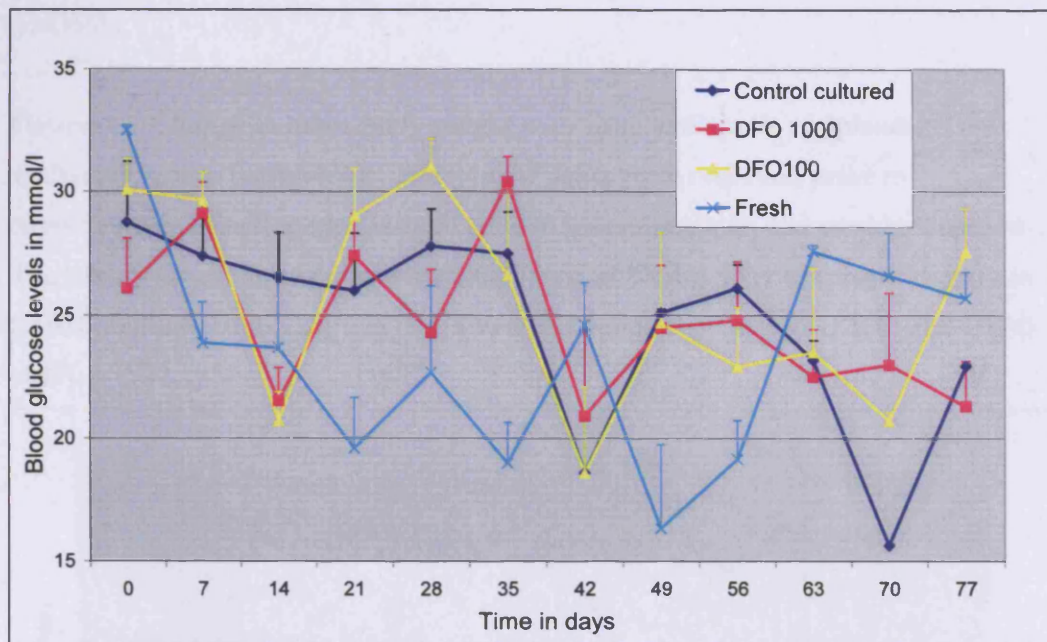


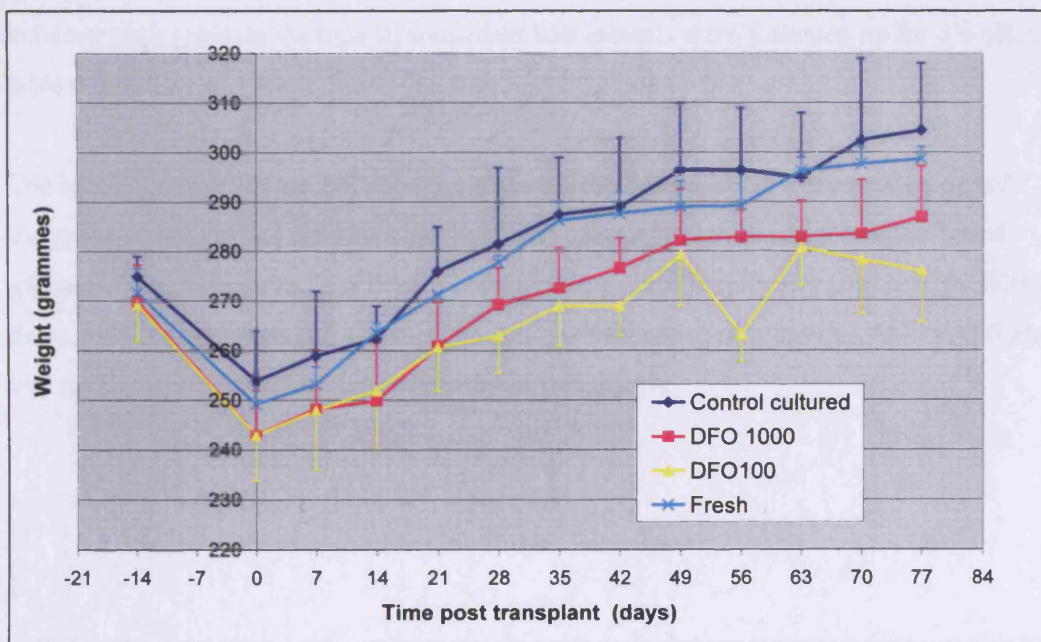
Table 6.2: Significances for the difference between transplanted groups in the change of blood glucose after transplantation, when analysed by repeated measures ANOVA.

	Fresh	Control culture	DFO 100
DFO 1000	0.007	0.884	0.65
DFO 100	0.019	0.745	
Control Culture	0.006		

6.2.2 Change in body weight after 350 islet transplant

All groups showed an increase in body weight from pre transplant to the day of sacrifice, table 6.1. The increase was significant in all groups, except the DFO 100 group. There was no difference in the change in body weight between any of the different groups (figure 6.2) and this was confirmed when the data was analysed by repeated measures ANOVA.

Figure 6.2: Change in mean body weight over time in diabetic recipients of 350 desferrioxamine treated islets. The body weights were recorded prior to streptozotocin injection (day -14), at time of transplantation and weekly thereafter. The islets were either transplanted fresh (control fresh), after overnight culture in CMRL alone (control cultured), or desferrioxamine treated, (DFO 100) and (DFO 1000).



6.3 Effect of desferrioxamine treatment on marginal (500 islet) syngeneic islet transplants

The 350 islet transplant experiments did not show any clinical advantage of using desferrioxamine treated islets over fresh or cultured islets. Other than non efficacy of the original hypothesis, one explanation could be the low dose of islets transplanted which is supported by the observation that no animals were rendered normoglycaemic in the fresh group. Therefore, the experiments were repeated with a higher marginal dose of islets in order to try to exclude the low graft mass for the lack of efficacy.

6.3.1 Change in blood glucose after 550 islet transplants

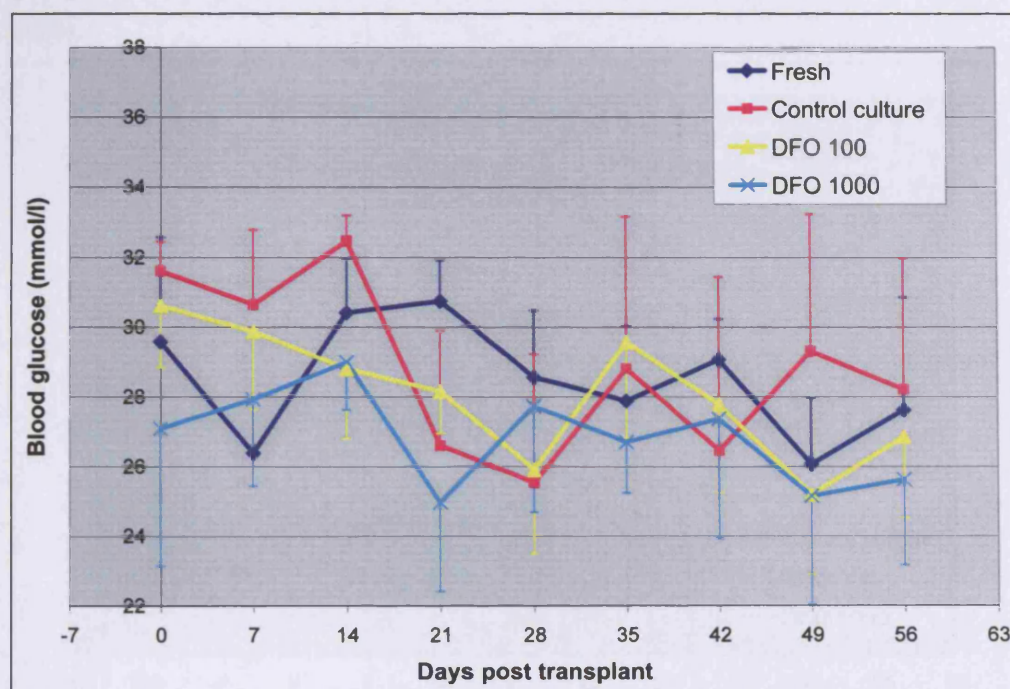
Three animals were rendered diabetic and transplanted in each group (fresh islets, islets cultured overnight in CMRL alone and islets treated overnight in either 100 μ M or 1000 μ M DFO). There was no significant difference in mean weight or mean blood sugar between each group at the time of transplant and animals were followed up for 8 weeks, table 6.3.

The blood glucose did not fall significantly over the 8 weeks post transplant in any of the groups. None of the animals were rendered normoglycaemic and the fall in blood glucose did not appear to be different between the groups graphically (figure 6.3). When the overall changes in blood glucose was analysed by repeated measures ANOVA, there was no significant difference between any of the groups.

Table 6.3: Change in body weights and blood sugar concentrations of diabetic rats on the day of transplant and day of harvest after transplantation with 550 desferrioxamine treated islets. Data is displayed as mean \pm 1 standard error.

	Pre transplant		Day of harvest		Significance of change between time points	
	Weight (g)	Sugar (mmol/l)	Weight (g)	Sugar (mmol/l)	Weight	Sugar
Fresh	236.7(3.8)	29.6 (3.0)	268.8 (7.0)	27.6(3.2)	0.020	0.11
Control Culture	261.3 (9.7)	31.6 (0.8)	260.9(28.6)	28.2(3.8)	0.497	0.31
DFO 100	272.7 (6.2)	30.6 (1.8)	306.1 (2.1)	26.9(2.3)	0.018	0.13
DFO 1000	241.3(10.1)	27.1 (3.9)	269.9 (9.4)	25.7(2.5)	0.002	0.11

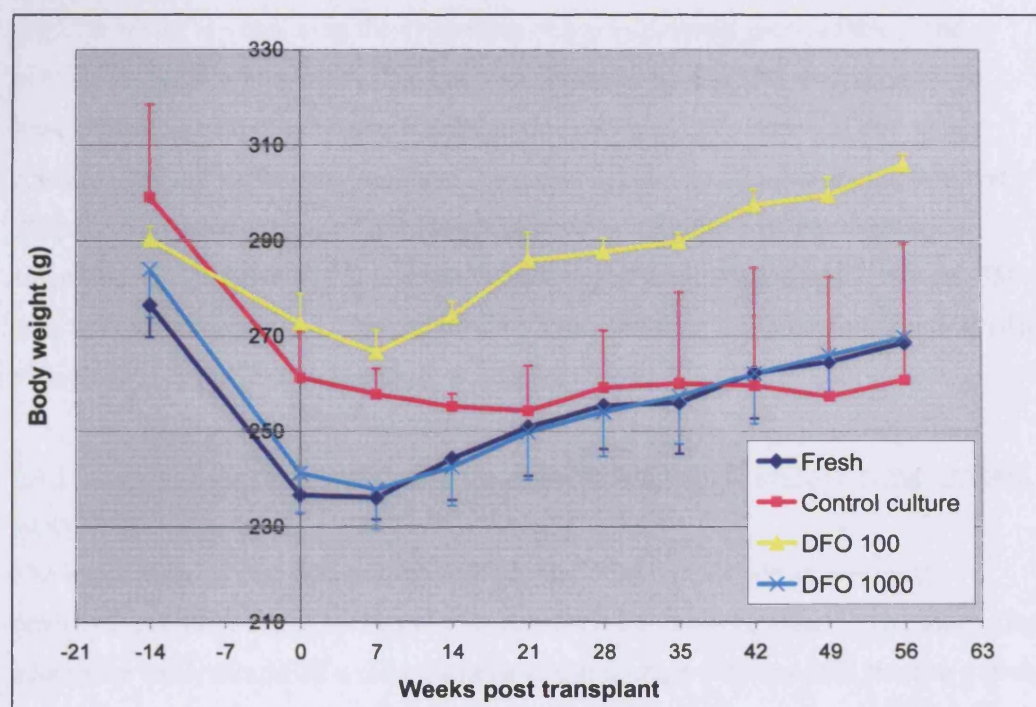
Figure 6.3: Change in mean non-fasting blood glucose over time in diabetic recipients of 550 desferrioxamine treated islets. The islets were either transplanted: fresh, overnight culture in CMRL alone (control cultured), or desferrioxamine treated (DFO 100) and (DFO 1000). Three animals were transplanted in each group.



6.3.2 Change in body weight after 550 islet transplant

All groups, except the control culture group, showed a significant increase in body weight from pre transplant to the day of sacrifice, table 6.3. When the changes in body weight between groups were analysed, there were no obvious differences (figure 6.4) and this observation was confirmed by ANOVA.

Figure 6.4: Change in mean body weight over time in diabetic recipients of 550 desferrioxamine treated islets. The body weights were recorded prior to streptozotocin injection (day -14), at time of transplantation and weekly thereafter. The islets were either transplanted fresh (control fresh), after overnight culture in CMRL alone (control cultured), or desferrioxamine treated, (DFO 100) and (DFO 1000).



6.4 Histological analysis of desferrioxamine treated and control islets post ultra-marginal (350 islet) transplants.

The changes in blood sugar and weight post transplant with desferrioxamine treated islets were disappointing, as it was expected to see improvement in outcomes with DFO treated islets. The principle behind the improved outcome was enhancement of islet engraftment by up-regulating the expression of key angiogenic growth factors and hypoxia preconditioning islets. The question remained whether the desferrioxamine treated islets had engrafted better but the crude endpoints of blood sugar and weight changes were not sufficiently sensitive to reveal the benefits of improved engraftment. Therefore, the islets were examined histologically to visualise any improvement in engraftment. This section will discuss the histological evaluation of islets from the 350 islet transplant recipients. Unfortunately, the livers were not available from the 550 islet transplant experiments due to storage mismanagement.

6.4.1 Gross histological appearance of transplanted control and desferrioxamine treated islets

The livers were harvested, fixed, embedded, sectioned and stained as previously described. All islets stained with the dual endothelial and insulin staining technique. The islets were easily identified within the hepatic architecture with one pole close to a portal triad. The islets from each experimental group had similar gross morphological appearances, vascular architecture and surrounding hepatic vacuolation, although there were some minor differences between the groups.

The fresh transplanted and DFO 1000 islets were often larger and easier to distinguish from surrounding liver, with better migration into the hepatic tissue and occasionally spanning from the portal triad to hepatic vein. Although the density of insulin staining is a poor endpoint for engraftment, there was a more homogeneous insulin staining pattern in the fresh and DFO 1000 islets compared with the very poor insulin staining in the control cultured islets. Representative images of the different groups can be seen in figures 6.5 to figure 6.8.

Figure 6.5: Dual stained islets from recipients of 350 fresh islets. Image a and b are of the same islet at 200x and 400x respectively. Image c and d are of additional islets at 400x magnification.

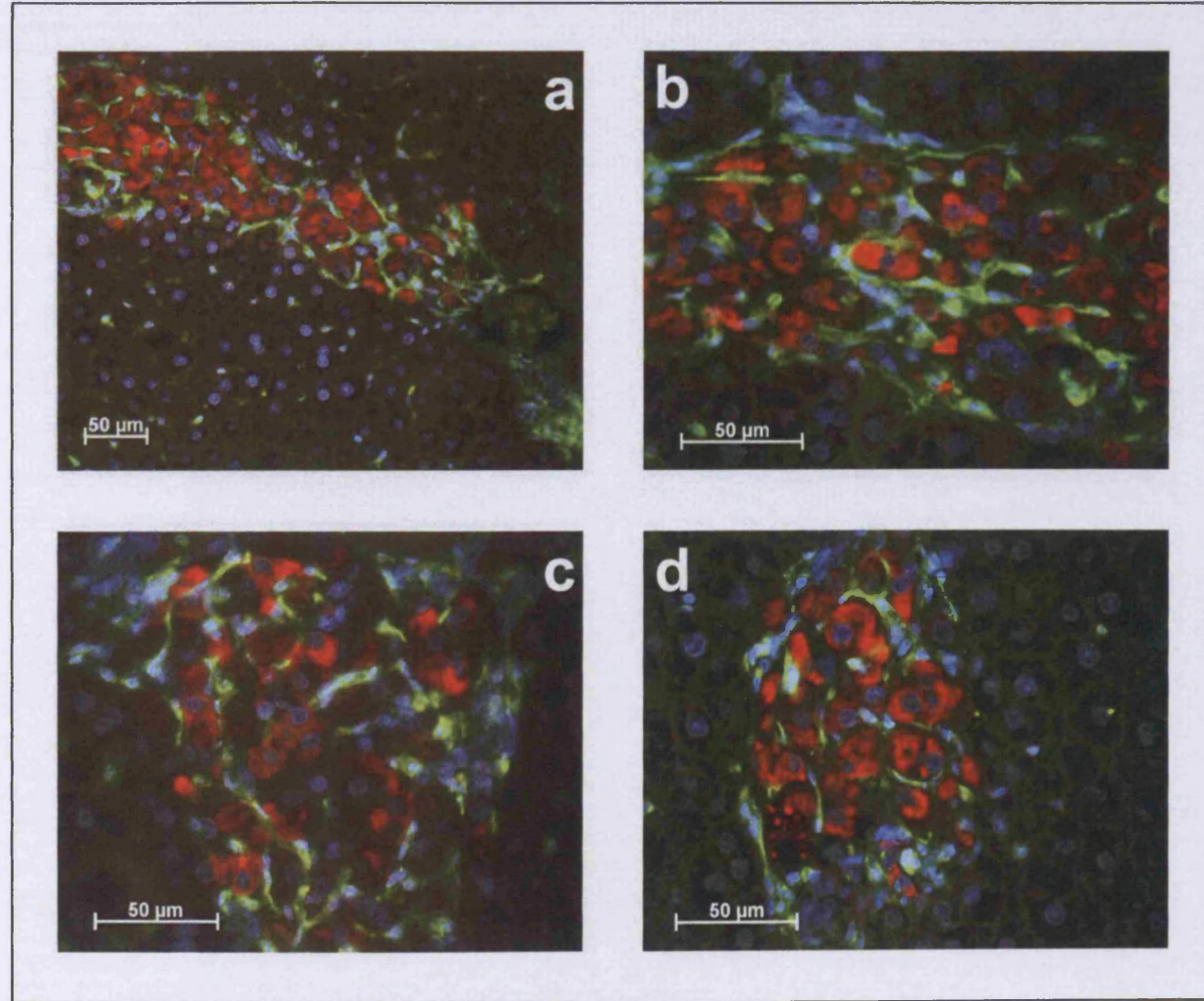


Figure 6.6: Dual stained islets from recipients of 350 control cultured islets. Image a and b are of the same islet at 200x and 400x respectively. Image c and d are of additional islets at 400x magnification.

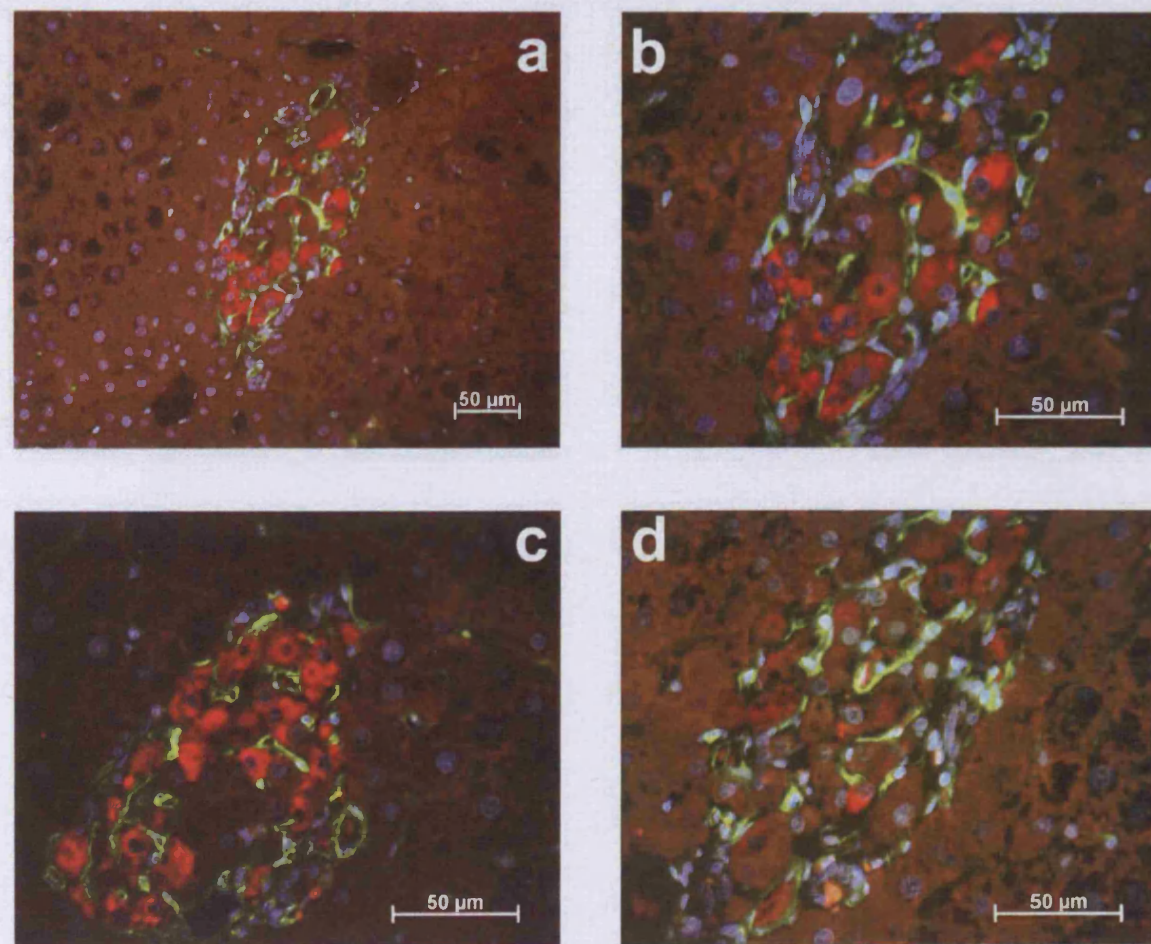


Figure 6.7: Dual stained islets from recipients of 350 DFO 100 islets. Image a and b are of the same islet at 200x and 400x respectively. Image c and d are of additional islets at 400x magnification.

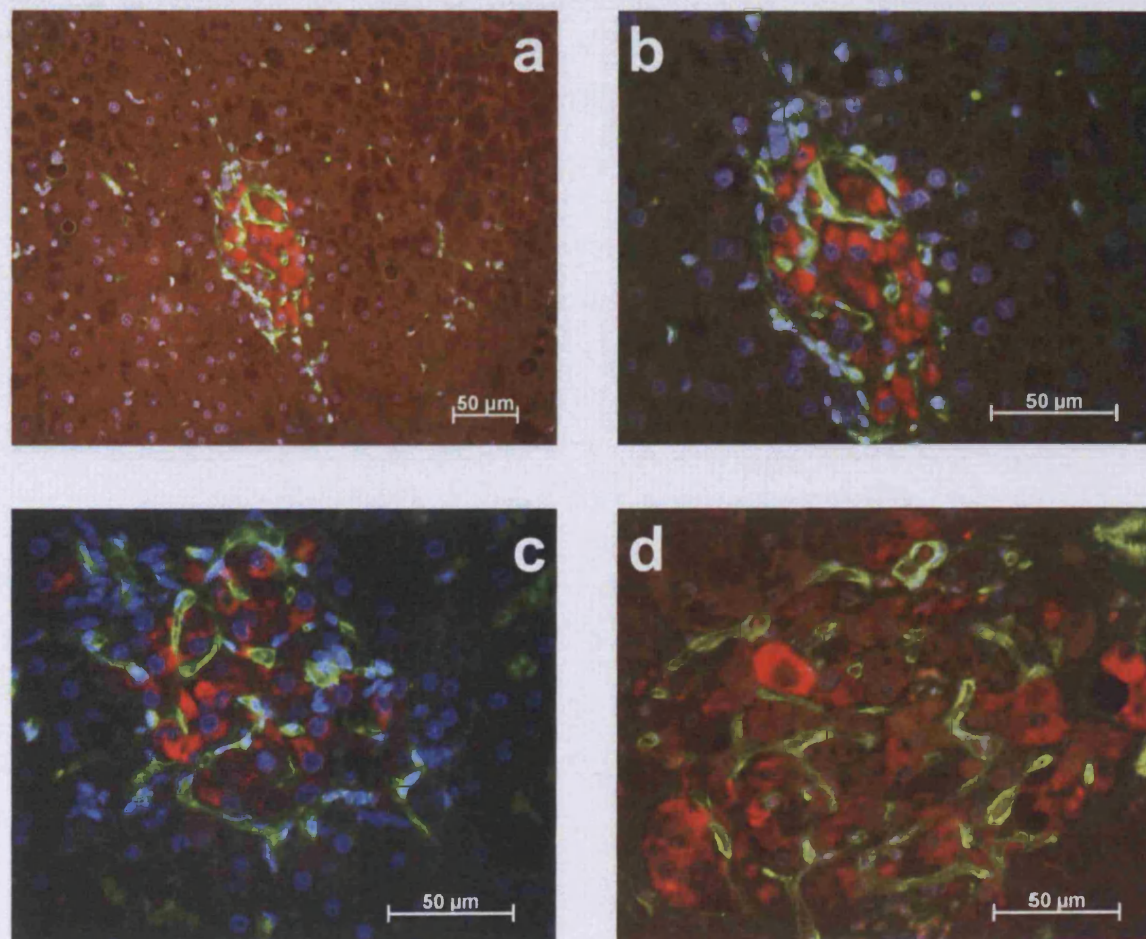
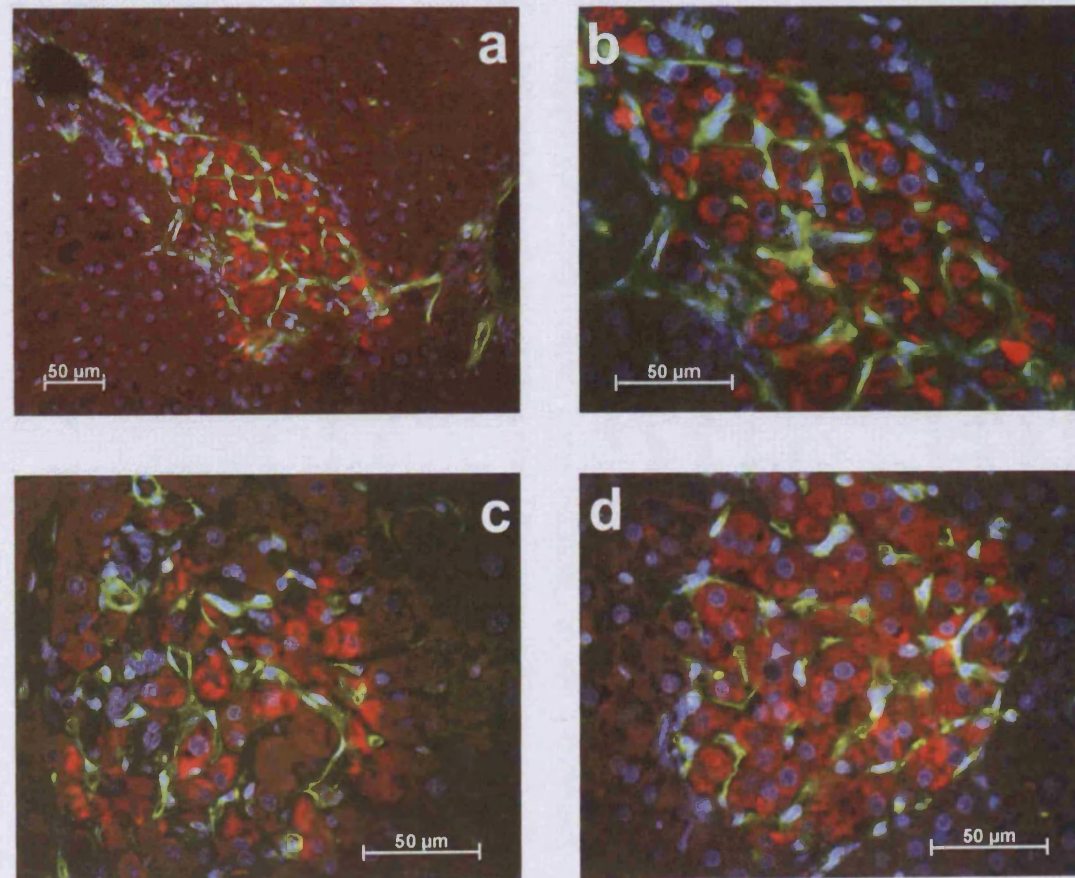


Figure 6.8: Dual stained islets from recipients of 350 DFO 1000 islets. Image a and b are of the same islet at 200x and 400x respectively. Image c and d are of additional islets at 400x magnification.



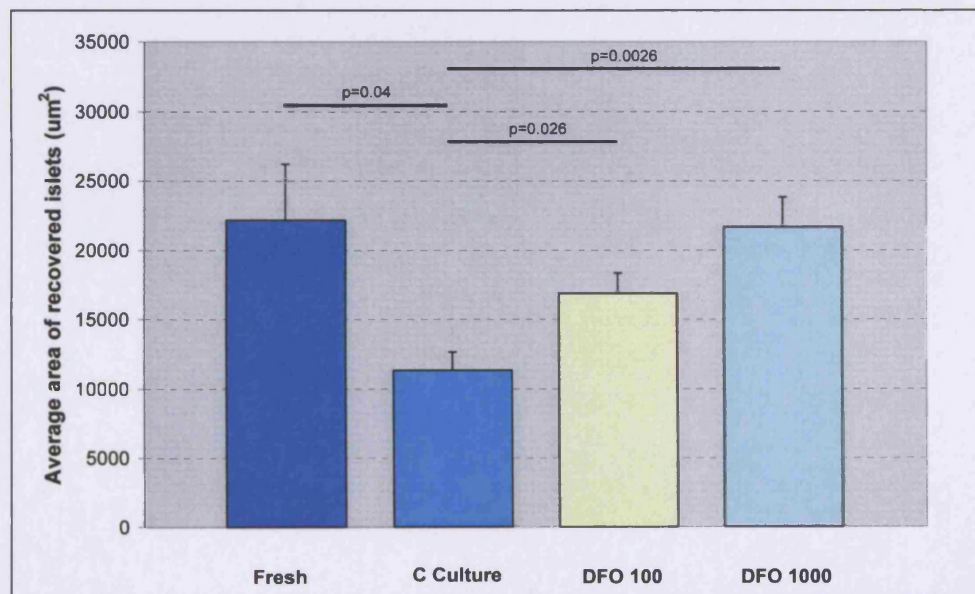
6.4.2 Quantitative histological analysis of desferrioxamine treated and control islets

The islet images were analysed as described in materials and methods. The islet area, vascular area and vascular density were calculated for all islet images. A total of 28 control fresh islets, 19 control culture islets, 32 DFO 100 islets and 40 DFO 1000 islets were suitable for analysis (of sufficient size with clear vascular network).

6.4.2a Analysis of islet area

The area of engrafted islets differed between all the groups, with the lowest islet area in the control cultured group and the highest islet area in the control fresh group (figure 6.9). The two desferrioxamine treated groups had lower islet areas than the freshly transplanted group, although not significantly so. There was significantly higher islet area in the desferrioxamine treated groups when compared to the control cultured group, suggesting that islet engraftment may be better in fresh and desferrioxamine treated islets compared to control culture islets.

Figure 6.9: Average islet area of control and desferrioxamine treated groups. The values are expressed as means \pm standard error.



6.4.2b Analysis of percentage vascular area and vascular density

All islets had roughly a quarter of their area dedicated to endothelium, with no significant difference between any of the groups and (Fresh- 23.2(\pm 2.6)%, control culture- 21.9(\pm 3.0)%, DFO 100- 24.8(\pm 2.4)% and DFO 1000- 22.5(2.0)%)- see figure 6.10.

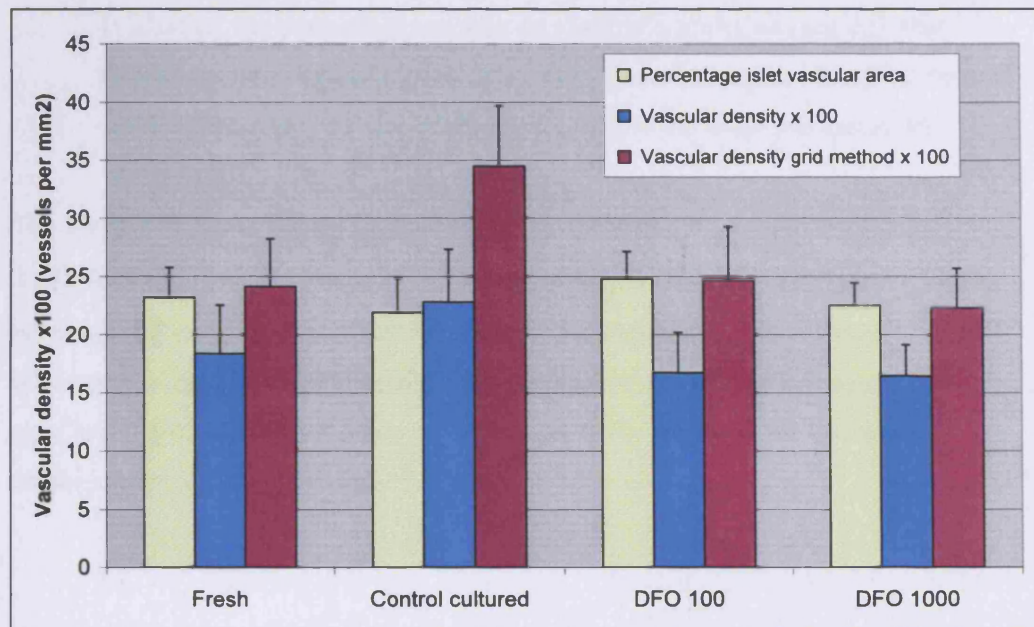
The vascular density was measured by both the number of vessel branches per islet area and the grid method. There was no significant difference in the vascular densities between the control fresh islets and the desferrioxamine treated islets. When the control cultured islets were compared to the other groups, there was a significantly lower vascular density in the fresh and desferrioxamine treated islets- see figure 6.10 (Vessel counting method: control cultured 2279 vessels/mm², fresh 1838 vessels/mm² (p=0.053), DFO 100 1674 vessels/mm² (p=0.003), DFO 1000 1645 vessels/mm² (p<0.001). Grid method: control cultured 3448 vessels/mm², fresh 2229 vessels/mm² (p<0.001), DFO 100 2468 vessels/mm² (p<0.001), DFO 1000 2411 vessels/mm² (p<0.001).

6.4.2b Analysis of percentage vascular area and vascular density

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The vascular density was measured by both the number of vessel branches per islet area and the grid method. There was no significant difference in the vascular densities between the control fresh islets and the desferrioxamine treated islets. When the control cultured islets were compared to the other groups, there was a significantly lower vascular density in the fresh and desferrioxamine treated islets- see figure 6.10 (Vessel counting method: control cultured 2279 vessels/mm², fresh 1838 vessels/mm² (p=0.053), DFO 100 1674 vessels/mm² (p=0.003), DFO 1000 1645 vessels/mm² (p<0.001). Grid method: control cultured 3448 vessels/mm², fresh 2229 vessels/mm² (p<0.001), DFO 100 2468 vessels/mm² (p<0.001), DFO 1000 2411 vessels/mm² (p<0.001).

Figure 6.10: Vascular densities (vessels per mm²) and percentage vascular area of transplanted control and desferrioxamine treated islets. Values are presented as means \pm 1 standard error.



6.4.2c Analysis of branching index

The vascular density estimation for the control cultured islets was different from the other groups. When this data is combined with a non significant difference in the percentage islet area dedicated to endothelium, it suggested that the architecture of the vascular networks formed may be different. Therefore, the branching index was analysed to estimate whether there were any differences in vascular architecture.

There was no significant difference in branching index between the groups, although the index in the fresh and DFO 1000 group was higher than that in the DFO100 and control culture group: fresh- 0.77(0.03), DFO 1000- 0.76(0.03), DFO 100- 0.69 (\pm 0.03) and control cultured- 0.69(\pm 0.06).

6.4.2d Analysis of transplanted islet vessel size

The vascular density results were surprising, as it was expected that the control islets would have a lower vascular density than the desferrioxamine treated islets. In addition, the percentage vascular area within the islet was not different between groups suggesting that there was a difference in the size of the formed vessels. Therefore, the size of the vessels within the islets was measured.

The average vessel width in the fresh islets was $4.69(\pm 0.09) \mu\text{m}$ and decreased to $4.33(\pm 0.09) \mu\text{m}$ ($p=0.007$) in DFO 1000 islets, $4.22(\pm 0.10) \mu\text{m}$ ($p<0.001$) in DFO 100 islets and $3.83(\pm 0.12) \mu\text{m}$ ($p<0.001$) in control cultured islets. When control cultured islets were compared to desferrioxamine treated islets, the differences were still significant ($p<0.01$) but there was no significant difference between the two desferrioxamine treated groups.

6.5 Discussion

The in-vitro experiments showed that desferrioxamine treatment of islets induced a prolonged over-expression of VEGF and it was postulated that the over-expression would lead to a faster normalisation of blood glucose with an associated improvement in islet engraftment. Unfortunately, the transfer of desferrioxamine treated islets to an in-vivo model did not obviously improve the rate of normalisation of blood glucose, the change in body weight or the achievement of insulin independence. Although the blood glucose data for the ultra-marginal dose transplants did suggest that there may be some benefit to transplanting islets fresh, the data was not reproduced in the marginal dose animals. Despite the two doses of islets used, none of the groups achieved insulin independence and the first issue to consider is whether the model was suitable for examining the hypothesis. This section will consider the suitability of the transplant model and the significance of the histological evaluation. The failure of the original hypothesis as a cause for the lack of difference between the groups will be considered in chapter 8.

6.5.1 Suitability of transplant model

The transplant model used should be a robust and reproducible experimental tool in order to examine any subtle changes in experimental conditions. Within each model there are a number of variables which may limit the accuracy of a particular investigation. The islet transplant model relies on the resolution of diabetes as the primary outcome and requires the induction of diabetes, measurement of blood glucose and transplant of sufficient β -cell mass to reverse the diabetes. Each of these processes are open to variability and this section will consider the different steps.

6.5.1a Induction of diabetes

Diabetes induction with streptozotocin is used as a gold standard for the study of islet transplantation. Streptozotocin is a nitrosurea derivative with broad spectrum antibiotic and antineoplastic activity which can induce diabetes at high doses (Rees and Alcolado 2005). Although STZ has a number of effects, including interference with glucose

transport, alkylation, induction of DNA strand breaks and alteration of glucokinase function, the induction of diabetes is probably a direct toxic effect on islets (Rees and Alcolado 2005). The single large dose administration of STZ in rodents induces a type 1 diabetic model but repeated low dose administration or in combination with high fat diet can induce a situation more like type 2 diabetes (Chen and Wang 2005).

In the present study, diabetes was induced prior to islet transplantation with the intraperitoneal injection of streptozotocin. Although most animals became diabetic within three days, some animals did not achieve the criteria for diabetes and some animals, mostly females induced with higher doses of STZ, became too unwell to transplant. This variability in diabetes induction could have led to a difference in the severity of diabetes and the dose of islets required to achieve normoglycaemia, thus increasing the intra-experiment variability. The reason for this variability is probably due to the route of administration of STZ and the nutritional status of the rat. The intra peritoneal administration of drugs is known to be more variable than the intravenous route (Sarangarajan and Cacini 1996) and it may have been more sensible to use intravenous administration of STZ. Secondly, the nutritional status and dose of STZ can effect the severity and type of diabetes (Chen and Wang 2005) and overnight starvation of rats prior to STZ can induce a more severe but more predictable diabetes model. Therefore, a lower dose of intravenous STZ could be administered after over night fast, in order to improve reproducibility further.

The use of spontaneously diabetic animals (Rees and Alcolado 2005) could have been considered but would have required a more complex model and a move away from syngeneic transplantation. Diabetic NOD-Scid mice have been used to receive islet transplants from humans (Sabek, Fraga et al. 2005) but the model requires kidney subcapsular transplantation and selective intra-portal transplantation would have been too difficult. The last option for diabetes induction would have been pancreatectomy but this technique is harder in rats, due to the diffuse nature of rodent pancreases, and would require two major procedures.

The model of diabetes induction used for these experiments was variable but other models would have been too technically demanding or not clinically relevant. It may have been possible to reduce the variability of diabetes by altering the route and conditions of STZ administration, therefore reducing experimental variability.

6.5.1b Measurement of blood glucose

The use of blood or plasma glucose as an endpoint for monitoring islets engraftment is widely accepted, as the reduction of blood glucose is a rough indicator of insulin secretion. Unfortunately, the upper range of blood glucose does not correlate well with the severity of induced diabetes and elevated blood glucose can correlate with a wide range of β -cell mass, depending on the size and nutritional status of an animal. Therefore, it is possible that there was a large variability of surviving β -cells at the time of transplantation despite the similarity of blood glucose estimations between animals. The survival of native β -cell mass has been shown to influence the outcome of experimental islet transplantation and animals with more surviving β -cells have better outcomes than animals with less β -cell survival (Hughes, Powis et al. 2001). Therefore, the variability of diabetes severity could have further influenced the reproducibility of the experiments.

The measurement of blood glucose is highly variable and will differ with the feeding patterns of the animals. Rodents tend to be nocturnal (or dark cycle) eaters (Strubbe and Woods 2004) and blood glucose measurement were performed at 11am each day in order to improve reproducibility. Nevertheless, animals showed daily variation in their blood glucose estimations which was particularly noticeable within the first two post operative weeks when the eating pattern of animals tends to be most disrupted (Sharp, Zammit et al. 2003). The first two weeks are the most crucial for islet transplantation as the islets engraft during this period and any difference in revascularisation caused by islet manipulation is hoped to be seen during this time. The variability of weekly blood glucose measurement was reduced by repeated measurements but there was still considerable variation which may have hidden any subtle changes in insulin responsiveness. These issues are difficult to overcome and while daily blood glucose

measurements may have reduced variation, it is difficult to justify starving the rats each evening and changing their feeding patterns in order to improve reproducibility.

Despite the difficulties with blood glucose monitoring, body weight is a sensitive indicator of animal well being. The average weights between the different groups were not significantly different and this confirms the fact that there was probably no difference between the experimental groups.

The most widely used method for reducing variability is to increase the number of experimental subjects. Only three transplants were performed in each group and while a higher number may have improved the statistical analysis, the large number of rodents required could probably not be justified given the lack of any difference when the initial experiments were analysed.

6.5.1c Islet dose

The marginal islet transplant model relies on a dose of islets which partially cures induced diabetes and achieves a situation where small changes in islet survival or engraftment lead to either persistent diabetes or normoglycaemia. If too small a dose is chosen, all animals will remain diabetic but too large a dose will render all recipients normoglycaemic with no discrimination between groups. The original experiments by Hughes et al within our laboratory showed that a dose of 500-700 non-selective intraportally transplanted islets in 280-300g recipients was sufficiently marginal to allow differentiation between experimental groups (Hughes, Davies et al. 2003). Our early experience with the selective right branch transplant technique suggested that the dose of islets required to induce insulin independence was less than that required for main branch transplantation. For this reason, the dose of 350 islets was initially chosen for transplantation.

After the first set of DFO experiments, it became clear that 350 islets were not sufficient to provide a marginal dose and all recipients remained significantly hyperglycaemic after transplantation. The dose of islets was increased further to 550 but there was still

no resolution of diabetes or discrimination between groups. On review of these results it became clear that the right branch transplant technique was not better at reversing diabetes and although 500 islets had proven to be a marginal dose in 200g female rats (Figure 3.2), this was not the case in 240-260g male recipients. Therefore, a larger dose of islets may be required for marginal transplantation in these recipients. Unfortunately, because none of the animals were rendered normoglycaemic, these results have neither proven nor disproven the hypothesis of whether DFO treatment improves the resolution of diabetes by a marginal dose transplant.

6.5.2 Histological outcomes

The clinical outcome data did not show any robust evidence for improved engraftment after DFO treatment. Therefore, islets were analysed histologically to examine whether DFO treatment and subsequent up regulation of VEGF had any effect on the morphology or vascular architecture of transplanted islets.

The islets analysed for histology were from the ultra-marginal (350 islet) transplants, as the marginal (550 islet) transplant livers were not available for analysis. The low dose of islets transplanted into the liver made their histological retrieval difficult and only small numbers of islets were analysed. The small number of islets analysed makes firm conclusions difficult but there were some striking qualitative and quantitative differences between the two groups.

The general appearance of fresh and DFO1000 islets was much better defined than the control cultured islets, with brighter insulin staining in the fresh and DFO treated groups. Although this is a relatively soft indicator of engraftment, the quantitative analysis also showed a significant difference in the islet area between the groups; with the highest area in the fresh islets and progressively lower average area in DFO1000, DFO100 and control cultured islets. This data suggested that the fresh islets had engrafted best and were followed by DFO1000, DFO100 and finally control cultured islets. These observations are in keeping with other studies which have examined increasing VEGF expression in transplanted islets, where VEGF up regulation has been shown to improve

the insulin staining (Zhang, Richter et al. 2004) and reduce islet mass loss after transplantation (Stagner, Mokshagundam et al. 2004; Chae, Lee et al. 2005; Lai, Schneider et al. 2005). The exact cause for these changes has not been fully examined but has been suggested to be due to an improved vascular supply caused by VEGF up regulation.

Unfortunately, this hypothesis does not explain why fresh islets engrafted better than the DFO treated islets, as the fresh islets would have had a lower VEGF expression at the time of transplantation. Other investigators have also shown improved engraftment in fresh islets compared to cultured islets (Olsson and Carlsson 2005) but could not correlate their findings with VEGF, as levels were higher in the cultured islets rather than the fresh islets. These findings suggest less of a role for VEGF and the authors postulated that the reduced engraftment in cultured islets may be due to loss of donor monocytes and endothelial cells during culture.

Vascular density has been used as a marker of islet engraftment with higher density suggesting better engraftment (Carlsson and Mattsson 1993; Carlsson, Palm et al. 2002; Mattsson, Jansson et al. 2002; Mattsson, Jansson et al. 2003; Olsson and Carlsson 2005). The increased expression of VEGF within islets has been shown to increase the relative vascular density and staining of endothelial markers in islets exposed to higher concentrations of VEGF (Zhang, Richter et al. 2004; Chae, Lee et al. 2005; Lai, Schneider et al. 2005). The data presented here is in contrast to these observations and suggests that the vascular density in control cultured islets is higher than that in fresh islets, with smaller vessels and no difference in the percentage vasculature. This result was unexpected as VEGF up regulation was predicted to increase endothelial migration and therefore increase vascular density.

One explanation for the higher vascular density in the control cultured group may be the time point of histological sampling. Most studies have investigated islet vasculature after 2-5 weeks but the recipients in this study were sacrificed after 11 weeks. Under these circumstances the differences seen between groups may have less to do with initial

engraftment and more to do with vascular remodeling. We know from our own and other author's data that remodeling continues long after the initial period of revascularization (Rooth, Dawidson et al. 1989). If cultured islets revascularise poorly and have lower oxygen tensions (Olsson and Carlsson 2005) they may continue to express angiogenic growth factors during the period of remodeling. The increased angiogenic growth factor expression would occur long after the well engrafted islets have stopped expressing these factors and therefore, the continuing hypoxia in cultured islets may stimulate further angiogenesis during remodeling and increase the vascular density in poorly engrafted islets. This situation may explain why the vascular density in our control islets is higher compared to the fresh or DFO treated.

The original rationale for treating islets with DFO was to speed up revascularization and improve reversal of diabetes with a marginal mass transplant. The simultaneous measurement of these two endpoints is difficult because the period of revascularisation is short (within the first week or two) and blood glucose does not tend to normalize for a few weeks. The time point of 11 weeks was chosen in order to allow the animals sufficient time to achieve normoglycaemia with the marginal mass transplants. This prolonged time point would have led to islets being examined well after revascularisation and during the remodeling phase of islet vascular architecture. Therefore, a better time point for examination of islets may be after 4 weeks to try to examine both endpoints or after 5 days to see whether desferrioxamine treatment accelerated revascularisation.

The engraftment and revascularization of islets is a complex process which requires the interaction of numerous growth factors, either previously defined or undiscovered. We have only looked at a small number of these growth factors which have been shown to be responsible for engraftment. Despite the changes in these factors with DFO treatment, the histological analysis suggests that DFO treated islets are more similar to the better engraftment seen in the fresh islets. This observation would suggest that the fresh islets probably engraft best but that DFO treatment may reverse some of the adverse effects of culture. If the adverse affects of culture are due to loss of endothelium, it is possible that

DFO may cause persistence of endothelium in culture by inducing cellular arrest (Hodges, Reese et al. 2005). Therefore, DFO treatment may have reduced the loss of islet endothelium associated with culture and may explain why the vascular architecture of desferrioxamine treated islets was more similar to fresh islets than that of control cultured islets. This observation would also suggest that islet revascularization has less to do with the effects of VEGF but may be influenced more by the persistence of donor endothelium.

The beneficial effects of DFO treatment are not purely limited to the up regulation of angiogenic factors and hypoxia preconditioning. Desferrioxamine has also been shown to modulate immune response and in-vivo, DFO can reduce the expression of MCP-1 (Bosco, Puppo et al. 2004) and VCAM-1 (Koo, Casper et al. 2003). Although this has less relevance to a syngeneic model, the immune modulation may be important for islet transplantation in an allergenic situation. Therefore, treatment of islets with DFO prior to allogeneic islet transplantation may provide some benefit and investigation of this clinically important model would be warranted.

6.7 Conclusions

These experiments were designed to bridge the gap between in-vitro experimentation and transfer to an in-vivo model of islet transplantation. Unfortunately, there was no obvious improvement in clinical outcomes when DFO treated islets were transplanted into syngeneic recipients. The lack of diabetes resolution in the control group suggests that the model may have weakened the conclusions drawn from the experiments and when combined with improvement of histological endpoints in DFO treated islets, suggests that further experiments are required to prove or disprove the hypothesis.

Chapter 7- Results:

Clinical islet transplantation

7.1 Introduction

Despite the early progress in non vascularised pancreas transplantation and the claim to the first xenogenic (Williams 1894) and allogenic transplants (Pybus 1924), the United Kingdom has lagged behind the rest of the world in the provision of clinical islet transplantation. Prior to the publication of the Edmonton protocol the UK had only performed 12 human allogenic islet transplants and none of the recipients had achieved insulin independence. The advances described in the publication from Edmonton encouraged Diabetes UK to form the UK islet transplant consortium and in early 2001, the head of research at Diabetes UK predicted that there would be 10 islet transplants performed in the UK by the end of the year. Sadly this prediction was not to come true and by March 2006, only eight Edmonton protocol transplants have been performed in the United Kingdom. This slow progress highlights the difficulties involved in transferring a successful technique to the practices and infrastructure of another country and the steep learning curved involved in isolating and transplanting pancreatic islets.

The advances of the Edmonton protocol have revolutionised the transplantation of pancreatic islets and rekindled the international interest in this valuable clinical tool. Despite these important advances, the transplantation of pancreatic islets requires further refinement before it is widely accepted as the treatment of choice for the difficult secondary complications of type 1 diabetes. Both whole pancreas and islet transplantation can improve metabolic control and abolish hypoglycaemia but islet transplantation currently requires a large number of donor organs to induce insulin independence (Shapiro, Lakey et al. 2000) and the long term graft survival rates are poor (Ryan, Paty et al. 2005). The issues surrounding these problems are multifactorial but are mainly reflected in the variability of islet yield and function after isolation (Lakey, Warnock et al. 1996; Goto, Johansson et al. 2005; Nano, Clissi et al. 2005). The number of pancreas preparations suitable for transplantation is often low and over 60% of

processed pancreases are not suitable for transplantation (Nano, Clissi et al. 2005). If islet transplantation is to be established as an accepted technique, the issue of variable islet yield needs to be addressed and the isolation technique optimised to achieve consistent improvements in islet yield.

After islets have been isolated from the whole pancreas they are transplanted into the portal vein of diabetic recipients. The common route of transplantation is percutaneous puncture of the liver with intra-portal catheterisation. Despite the improvements in radiological techniques, there is still a significant risk of portal thrombosis and haemorrhage from the liver (Shapiro, Lakey et al. 2000; Ryan, Paty et al. 2005). This significant peri transplant risk is combined with the long term adverse effects of immunosuppression and will require further improvements to reduce the negative impact of islet transplantation.

The establishment of new islet transplant programmes throughout the world have a dual role in providing clinical care to diabetic recipients. The first and most important role is to establish a functioning clinical programme with repetition of the Edmonton experience. This usually requires duplication of the original protocols in order to avoid changing any key procedures of unknown significance which may impact on final outcome. Once a programme is established, the protocols should be further refined to optimise islet transplantation and provide an improved clinical procedure.

As part of my PhD studies I contributed to the clinical islet transplant programme at the Royal Free hospital and extended my experience of transplantation into the field of clinical islet transplantation. This provided a natural link from the basic science work through to the end result and challenge of clinical islet transplantation. I was heavily involved in the isolation, culture and transplantation of human islets as well as taking a major role in the work up, peri- and post transplant care of islet transplant recipients. This chapter documents some of the challenges and successes of establishing a clinical islet transplant programme within the United Kingdom and emphasises areas in which we have optimised the original protocol.

7.2 History of the Royal Free clinical islet transplant programme

The clinical islet transplant programme at the Royal Free hospital was established in late 2000 and started to receive human pancreases for islet isolation from February 2001. After mastering the basics of islet isolation and purification, the isolation process was transferred to an MHRA accredited laboratory in April 2003. Ethical permission for transplantation was granted in October 2003 (see Appendix 3 for ethics submission) and the isolation process was further refined and validated to product release criteria over the next two years. In March 2005, the first patient underwent islet transplantation and subsequently received a second infusion of islets in July 2005. In addition, a second patient was transplanted in May 2005 and a third in August 2005.

7.3 Donor pancreases for islet isolation

From February 2001 until the end of August 2005, 76 pancreases were offered to the programme at the Royal Free hospital (figure 7.1). Initially, the number of offers received was low (0.3 pancreas offers per month in 2001) but after a number of meetings and information sharing sessions with donor coordinators, the number of offers started to increase (1.8 offers/month in 2002, 1.3 offers/month in 2003, and 1.5 offers/ month in 2004). Despite the increase in offers, the supply of donor organs was still slow and it was not until after our first transplant that the number of offers started to increase further (2.3 offers/ month in 2005 and 3.25offers/ month since the first transplant). The increased number of offers received subsequently highlighted the logistical problems of lab turnaround time and restocking. On the contrary, the increased supply allowed selection of donors for transplantation and acceptance of only good pancreases which resulted in a total of 15 pancreas offers being refused (figure 7.2).

Figure 7.1: Average number of pancreas offers per month to the Royal Free islet isolation unit between November 2000 and August 2005. The black line represents the trend in the number of offers per month.

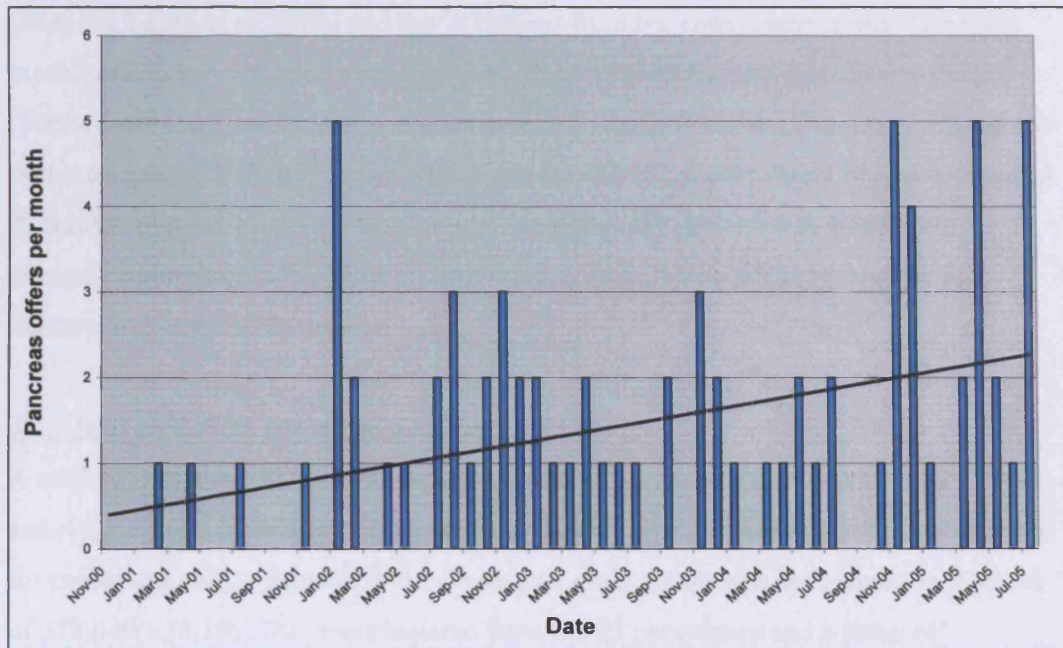
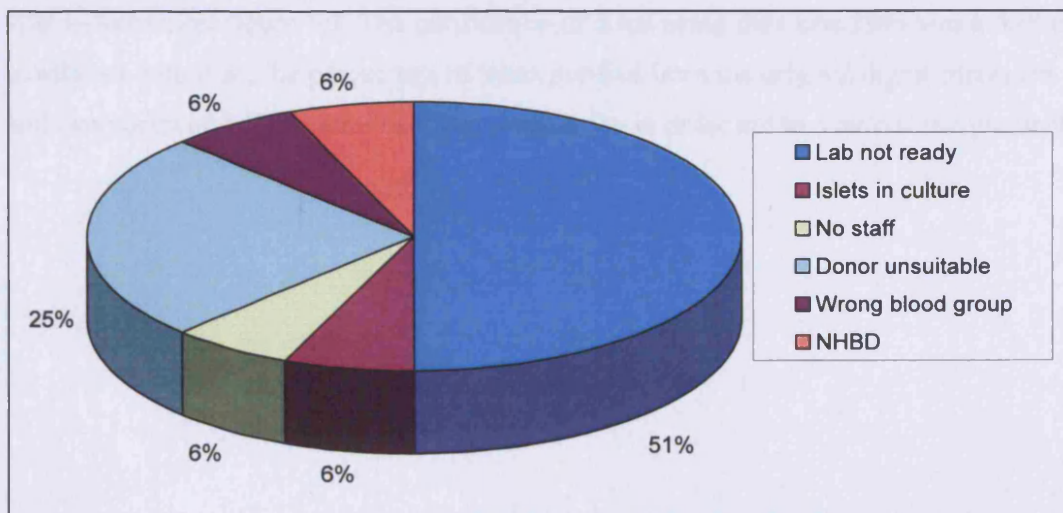


Figure 7.2: Reasons for refusal of pancreas offers to the Royal Free islet isolation unit.



7.4 Pancreatic islet isolation

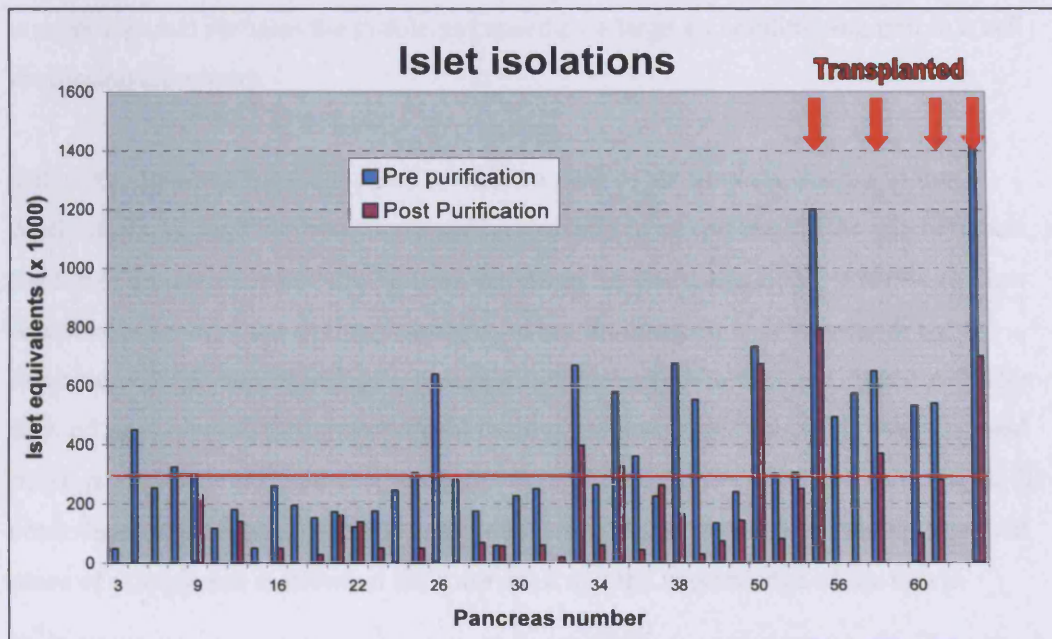
Islets were isolated using a modified version of the automated technique (Ricordi, Lacy et al. 1988) which incorporated some improvements from the Edmonton protocol (Shapiro, Lakey et al. 2000) and modifications from our own observations. The main modifications we instituted were to use a collagenase and neutral protease mixture (Serva, Germany), rather than using Liberase HI (Roche Pharma, Germany) and run the digest temperature slightly lower with a second coil to cool the digest before harvesting. A number of other small changes were made within the process which included automated perfusion rather than syringe loading and DNase washing, and are too numerous to describe in detail.

7.4.1 Islet yield from processed pancreases

A total of sixty three pancreases were accepted and processed between February 2001 and August 2005. A number of pancreases, roughly five, were received but not suitable for processing due to damage to the organ (the exact figure was not recorded). A mean of 378,049(\pm 38,796) IEQ were isolated from the 63 pancreases and a mean of 200,105(\pm 39,030) IEQ were purified from 29 of these preparations suitable for Cobe purification.

The initial isolations were poor and there was a steep learning curve for the technique of islet isolation, see figure 7.3. The purification of islets using the Cobe 2991 was a further hurdle but with time, the percentage of islets purified from the original digest increased and two purification runs were used per preparation in order not to overload the gradient.

Figure 7.3: Islet equivalent yield from pancreas preparations over time. The red line is an arbitrary cut off point of 300,000IEQ for the minimum transplantable yield.



7.4.2 Cooling of the Cobe 2991 centrifuge

The Cobe 2991 centrifuge, used to purify islets from the pancreatic digest, was designed 20 years ago and was not originally intended to be refrigerated. The purification process is lengthy, roughly 20-25 minutes, and involves the use of non physiological density gradients with high osmolarity. During this process, islets are re-warmed and have an increased risk of enzymatic digestion from the proteolytic enzymes released from the exocrine fraction of the pancreas. The perceived benefit of cooling the digest during purification is a reduction in the proteolytic digestion of islets by exocrine enzymes and a reduced metabolism of islets while they are surrounded by the harsh environment of the ficoll density gradient. Therefore, cooling of the digest may help reduce the damage and cellular compromise during density purification and therefore improve islet health and viability.

Professional refrigeration modifications are not only expensive (between US \$5,000 – 20,000) but may exceed the specifications of a class 1 cell separation unit. Therefore, a method was developed for cooling the Cobe 2991 using dry ice, which is relatively inexpensive and obviates the problems caused by a large air conditioning unit in a cell processing laboratory.

Initial inspection of the Cobe 2991 revealed a flow of air from the bottom of the machine, up through the bowl surrounding the rotor head and out via the gap between the top of the machine and the Perspex covers to the centrifuge bowl. It is this air flow that warms the machine during operation, as the air is drawn in from outside the machine over the hot centrifuge motor and subsequently into the centrifuge bowl. This flow of air is created by the centrifugal force generated by the spinning rotor head and initial modifications aimed at limiting the flow of air, in order to reduce warming of the centrifuge head (figure 7.4). The upwards flow of air was limited by placing a pre cut piece of polystyrene in between the rotor shaft and the bottom edge of the bowl.

Cold air was generated by passing air over carbon dioxide ice placed on a metal mesh within a piece of four inch plastic ducting. The mesh and tubing was attached to a hole in the Cobe lid above the centrifuge rotor head and the air flow was assisted by the use of a toilet extractor fan. The Cobe lid was further adapted by drilling a hole in the top and inserting a temperature probe into the centrifuge bowl, in order to monitor the air temperature within the centrifuge bowl.

The initial prototypes worked well but the temperature only reached 10-15°C and the carbon dioxide ice sublimed rapidly. The prototype was then modified further by sealing the gap between the lid and body of the centrifuge, to limit loss of cold air, and adding a second large hole to the centrifuge lid in order to recirculate the cold air and maintain the low air temperature. The two holes in the lid were placed at different distances from the rotational axis of the centrifuge head in order to benefit from the flow of air generated by the centrifugal force of the rotating centrifuge head (the inflow hole was close to the rotating axis and outflow hole was further from the rotational axis).

These modifications improved the cooling device further and experiments, using saline loads onto the Cobe, proved that an air temperature of 0°C within the centrifuge bowl could be achieved, with fluid offloading temperatures of 10-12 °C. The device was further modified with a fan rheostat added to adjust the airflow across the dry ice and control the air temperature within the centrifuge bowl – figure 7.5.

Experiments were subsequently performed on human islet preparations with pre-cooling of the centrifuge, followed by gradient and digest loading. Measurements were recorded of the air temperature within the centrifuge bowl, the temperature of the digest at loading, the temperature of the digest at offloading and the temperature of the bag after the purification run. Four runs with and four runs without the device were used- figure 7.6.

The room temperatures were not significantly different between the non cooling and cooling runs, 24.03°C vs. 23.97 °C ($p=0.26$) respectively. In the non cooling run, the Cobe air temperature during operation was slightly but not significantly higher than the room temperature, 24.02 °C vs. 26.44 °C ($p=0.38$) respectively. In the cooling runs, the Cobe air temperature during operation was significantly lower than room temperature, 23.97 °C vs. 0 °C ($p<0.001$). The digest loading temperatures were not significantly different between the non cooling and cooling runs, 13.28 °C vs. 12.93 °C ($p=0.213$) respectively. In the non cooling runs, the digest temperature increased significantly during purification from 13.28 °C to 28.76 °C ($p<0.001$), which was also significantly higher than the Cobe air temperature ($p>0.001$). In the cooling runs, the digest temperature fell significantly during purification from 12.93 °C to 10.4 °C ($p=0.033$).

This data suggests that during the purification process, the temperature of the Cobe 2991 machine increases without cooling. This increase in temperature, probably due to the heat generated by the by the motor and directly transferred up the shaft to the rotor head, increases the temperature of the digest during purification to a level above room temperature (28.76 °C). At this temperature, it is possible for exocrine enzymes to be activated and damage the islets within the preparation. The addition of the cooling

device not only stopped this warming effect but reduced the temperature of the digest during purification. Unfortunately, viability and islet function is not available and the number of runs was too small to tease out any difference in yield during purification. In addition, concentrations of carbon dioxide in the islet isolation facility were not measured during the purification process.

Figure 7.4: Schematic diagram of the cooling device for the Cobe 2991.

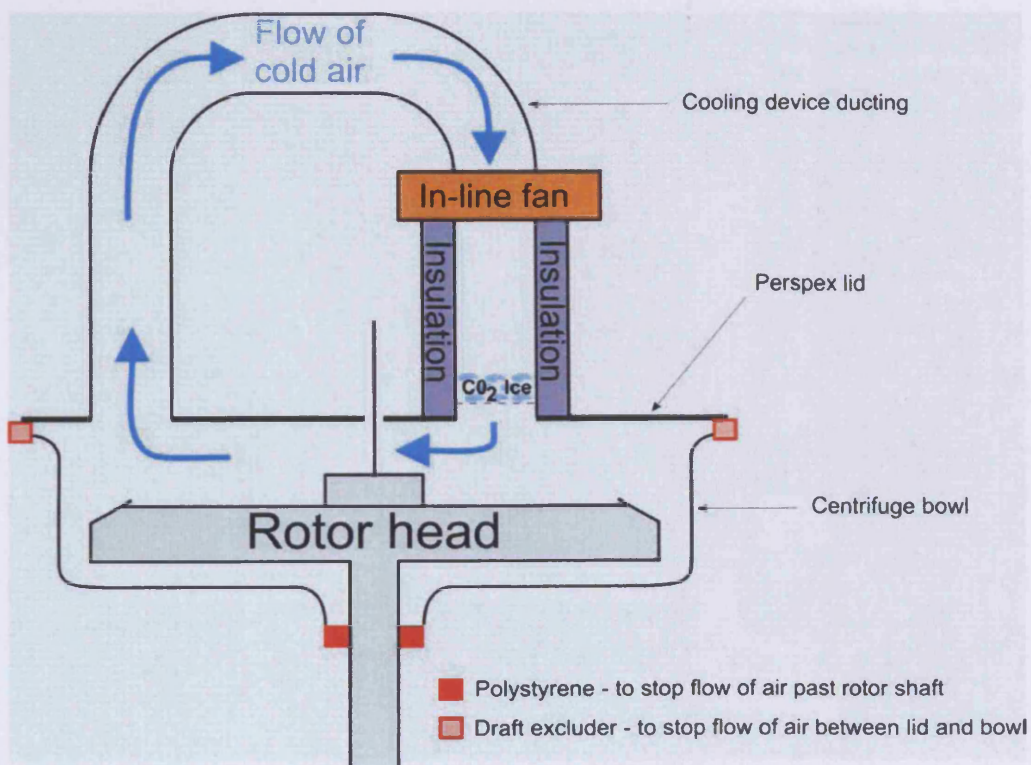
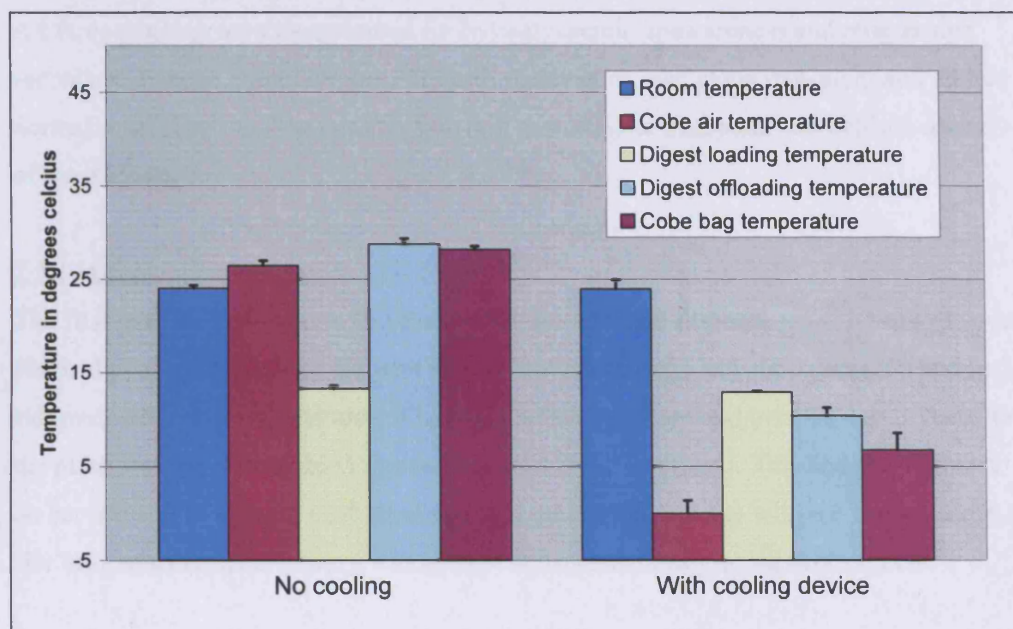


Figure 7.5: Final prototype of the cooling device used for refrigerating the Cobe 2991 centrifuge. The tubing is constructed from 4 inch ducting with the addition of a toilet extractor fan. Toilet flanges have been added to aid the removal of the top “u-bend” connector. The down pipe, containing dry ice, has been insulated by using a surrounding piece of 6 inch drainage pipe and cavity filler. The fan rheostat was added to control the flow of air across the dry ice and a temperature probe was added to measure the air temperature within the centrifuge bowl. The draught excluder can be seen under the Perspex lid.



Figure 7.6: Temperatures achieved in the digest and centrifuge bowl with and without the cooling device. Values are presented as mean \pm standard error.



7.5 Clinical islet transplantation

Three patients underwent clinical islet transplantation, in the period until January 2006. All three patients were transplanted for hypoglycaemic unawareness and progressive secondary diabetic complications. They all received an islet alone transplant and all had normal renal function. The next section will describe the transplant and clinical course of the patients.

7.5.1 Islet recipient number 1

The first islet recipient was a 39 year old female who had diabetes since the age of seven. She had poor control of her diabetes throughout the teenage and early adult life and had suffered with recurrent episodes of hypoglycaemic unawareness over the last 3 years, to the point that she was unable to go to the shops unaccompanied. This had great impact on her life and as a single mother, compromised her relationship with her two children. Her only other medical history was of hypothyroidism on thyroxine replacement.

On the third of March 2005, she underwent intra-portal islet transplantation and received 660,000 IEQ (12,200IEQ/Kg) of 88% viability. Portal puncture was uncomplicated and portal pressure rose to a maximum of 9mmHg. The initial post transplant course was unremarkable and she was discharged 4 days after the procedure. Her blood glucose concentrations did not drop rapidly but after two to three weeks her glycaemic variability started to reduce. Interestingly, she started to regain sensation of hypoglycaemic episodes within the first week and, although the symptoms were not similar to her previous hypoglycaemic warning, she was able to recognise hypoglycaemia again. Subsequently, her episodes of hypoglycaemia became much less frequent and she did not require any outside assistance.

Within two to three days of her transplant she developed abdominal discomfort which consisted of abdominal fullness and dull ache in her epigastric region. The discomfort was associated with a rise in liver enzymes which peaked at an ALP of 130U/l, AST of 308U/l and ALT of 222U/l. The clotting and other markers of synthetic function were not affected. The pain and abnormal liver function tests resolved by the second week

post transplant. By day 5 post transplant the white blood cell count started to fall and septrin was stopped. The white blood cell count fell to $3.53 \times 10^9/l$ after two weeks post transplant and slowly rose after that time.

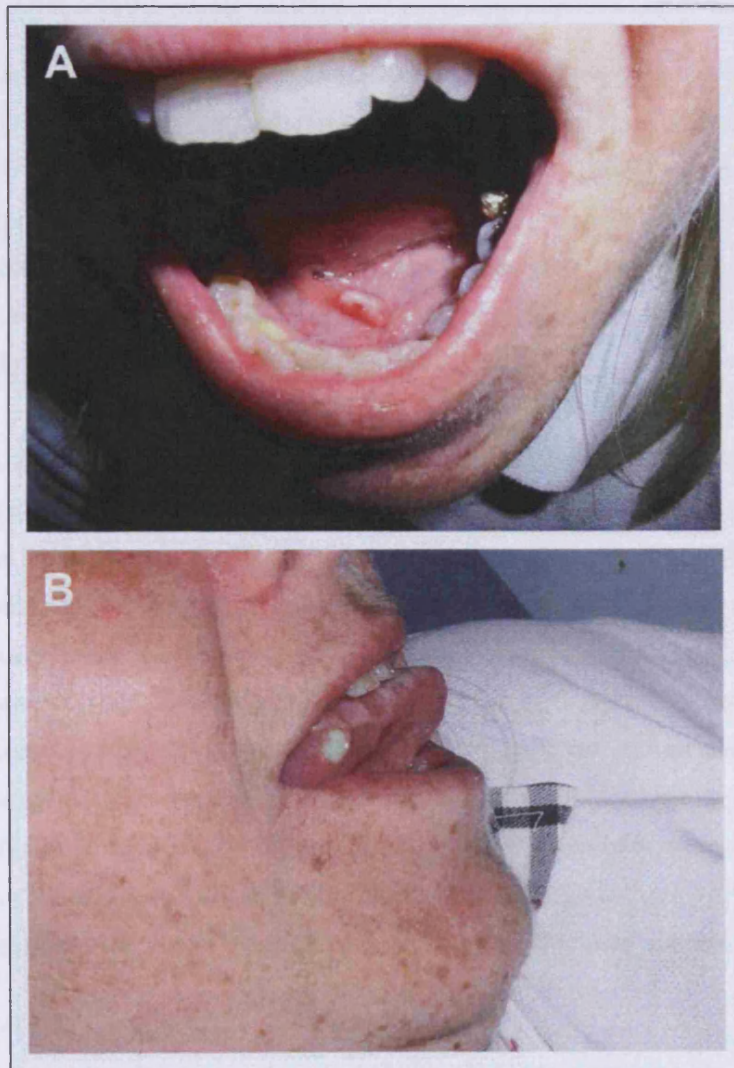
Oral ulceration was a significant side effect in this recipient (figure 7.7) and she started to have difficult oral ulcers from one month post transplantation. The ulcers were initially treated with antiseptic and anaesthetic mouthwashes but this did not improve her symptoms. Advice was sought from the otorhinolaryngologists who suggested doxycycline mouth washes, as used for difficult recurrent aphthous ulceration in unimmunosuppressed patients. Not only did this not relieve her symptoms but she swallowed the mouthwash which led to inhibition of the metabolism of her immunosuppressants and subsequent high trough levels of the drugs. She was then switched to regular, three plus times per day, tooth brushing and topical steroid application. This settled her symptoms but she still had further ulcers. Swabs of the ulcer resulted in the isolation of herpes simplex virus 1 (HSV1) and she was subsequently treated with oral valacyclovir. At the same time, we stopped her atorvastatin, as there were reports that this drug exacerbated oral ulceration. The ulcers disappeared within one day but after the course of valacyclovir was finished, she was again troubled with oral ulcers. This culminated in an episode of oral ulceration with subsequent presumed bacterial sepsis, where the only identified portal of entry for infection was the ulcer (figure 7.7B).

The patient also developed further infections in her chest and ear. The ear infection was a particularly severe form of otitis externa (malignant otitis externa) which was caused by a *Pseudomonas Aeruginosa* infection and required inpatient stay and intra-venous antibiotics. During this time, her immunosuppression was stopped due to life threatening infection but was restarted on resolution of infection and after discussion with the patient.

The metabolic control improved but not dramatically. Her blood finger prick tests improved to readings of 8-9mmol/l from pre-transplant levels in double figures and glycated haemoglobin fell from 12.2% to 7.8%. Despite these improvements, her insulin

dose was relatively unchanged and basal C peptide levels were only just recordable at 98pmol/l and 128pmol/l. Despite the recurrent infections, the patient felt her life had been revolutionised and wished to continue immunosuppression. In view of the marginal results from her islet graft, she was re-listed for transplantation.

Figure 7.7: Oral ulceration in islet recipient 1. Image A) shows an aphthous ulcer on the base of the mouth under the tongue and closely associated with the submandibular salivary duct. Image B) shows an ulcer on the left lateral aspect of the tongue with a green discoloured base which is probably secondarily infected.

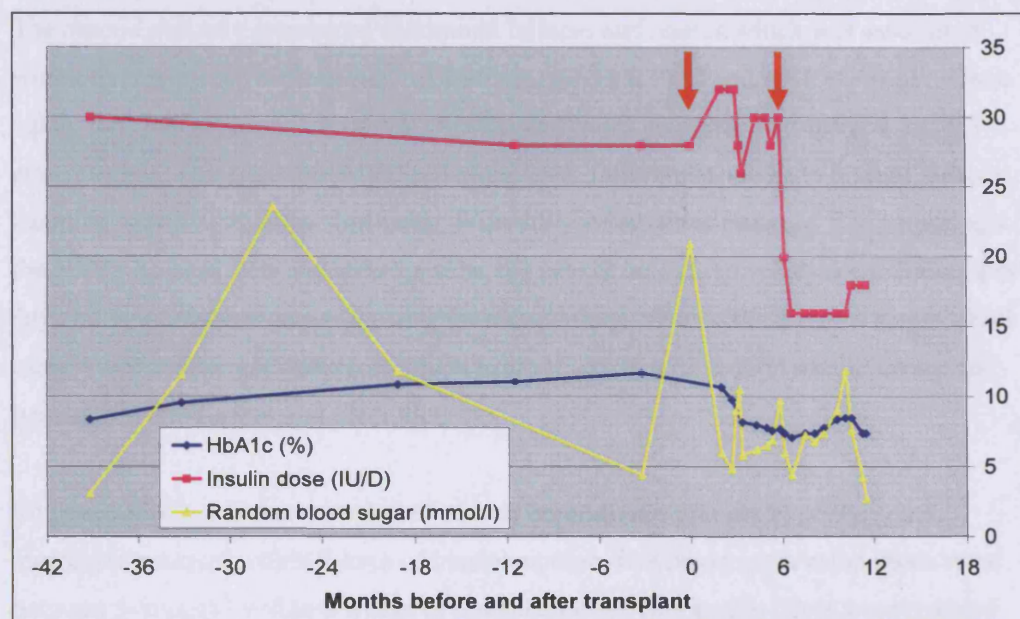


On the 26th of July 2005, she underwent a second islet transplant carried out at the Churchill hospital in Oxford. The transplant was carried out in Oxford as the unit is part of a combined network with the Royal Free (FROX network). She received 280,000IEQ (5200IEQ/Kg) of 95% viability. The transplant was associated with difficult access to the portal vein but there was no significant procedural complication. Her blood sugar estimations improved almost immediately and improved to 5-7mmol/l within days of the transplantation and she had no further episodes of hypoglycaemia. Initial insulin doses were unchanged but gradually reduced to almost half her pre-transplant dose within 2 months of her transplant.

Her subsequent course has been relatively uncomplicated although she has had further trouble with aphthous ulceration, acneform eruptions and an infected boil on her face. She also suffered a single episode of graft dysfunction in association with low levels of immunosuppressants which responded to increased doses of insulin and tacrolimus. As of January 2006, she has well controlled blood sugars between 5-8 mmol/l, with a glycated haemoglobin of 7.4% and basal C peptide of 362pmol/l. Some of her biochemical parameters are outlined in figure 7.8.

The impact of the transplant was not only observed in the biochemical parameters and hypoglycaemic episodes. Due to her severe symptoms prior to the transplant, the recipient had formed a carer/dependent relationship with her partner. This was not obvious prior to the transplant but her partner would supervise her glycaemic control and check she was not hypoglycaemic during trips out of the house. As the patient was unable to go to the shops for fear of hypoglycaemia, the supervision was continuous and the patient relied heavily on her partner. After the transplant, the patient was able to pursue a normal life and started to socialise with her friends without the need for her partner to supervise her diabetes. This led to her partner losing his carer role and his attempts at supervision were deemed interfering. This situation led to the breakdown of the relationship and highlights the social and psychological impact of transplantation.

Figure 7.8: Selected parameters from recipient 1. The graph shows values for random blood glucose, glycated haemoglobin and insulin dose per day over time before and after transplantation. The red arrows indicate the timing of islet infusions.



7.5.2 Islet recipient number 2

The second recipient was a 52 year old female who had diabetes since the age of 18. Her control had been very erratic during her early twenties and she had subsequently developed background diabetic retinopathy and diabetic gastropathy. The variable emptying of her stomach and diarrhoea had made sugar absorption erratic which further complicated the timing of her insulin injections. Over the five years prior to her transplant she had reducing awareness of her hypoglycaemic episodes which eventually culminated in full blown hypoglycaemic unawareness.

On the 20th of May 2005, she received a single islet infusion of 360,000IEQ (6200IEQ/Kg) of 90% viability. The digest had an estimated purity of 40% and volume

of 9ml. The transplant was performed under radiographic guidance and portal pressure rose from 4.5mmHg to 16mmHg. The procedure was uncomplicated and the patient was discharged after 4 days.

The second patient experienced abdominal fullness and nausea which was associated with a liver enzyme rise to at ALP of 264U/l, AST of 627U/l and ALT of 476U/l. Once again, the total white cell count fell after the first week and bottomed out at $2.3 \times 10^9/l$ after 11 days. This time, the white cell count took 5 months to return to normal, despite stopping septrin within the first week. With the lessons learnt from our first patient and the HSV1 isolated from the mouth swabs, the patient was given valacyclovir 500mg per day and mouthwashes as prophylaxis for mouth ulcers. She suffered with a single small ulcer 4 weeks after her transplant which healed quickly with topical steroid cream and has not had any further oral ulceration.

The control of her diabetes improved almost immediately and her hypoglycaemic awareness returned within 7 days of transplantation. Her blood sugar estimations were between 5-8mmol/l within 4 weeks of transplantation. Her insulin doses were reduced after two weeks and she stopped all soluble insulin two months after transplantation. As of January 2006, she takes 4 units of glargine per day and on occasions has forgotten to take the insulin but has achieved normal glucose estimations for the following days. The long acting insulin has not been stopped as it is hoped that the basal insulin will reduce the metabolic stress/glucose toxicity to the islets and hopefully prolong the life of the graft. The basal C peptide was positive within one month of transplantation at 333pmol/l and has maintained around 300pmol/l on further sampling. Some of the metabolic parameters are outlined in figure 7.9.

In addition to the single mouth ulcer and leucopenia, the second recipient has two episodes of cellulitis following trauma, one after an iron burn and one after a cut to the leg. The only other potentially more serious complication relates to her renal function. At the time of transplantation the patient had a creatinine of 85 μ mol/l (correlating to an estimated GFR by Cockcroft and Gault formula of 60ml/min) and a normal albumin

Figure 7.9: Selected parameters from recipient 2. The graph shows values for random blood glucose, glycated haemoglobin and insulin dose per day over time before and after transplantation. The red arrow indicates the timing of her islet infusion.



7.5.3 Islet recipient number 3

The third recipient was a 49 year old female who had developed diabetes at the age of 16. Her control was generally good but she suffered with diabetic retinopathy which required laser treatment and autonomic neuropathy which manifested itself with postural hypotension (ECG at the time of work up showed no R-R variation with forced respiration). She also suffered with hypoglycaemic unawareness which limited her daily activities and subsequent metabolic control was poor due to fear of hypoglycaemia. Independent of her diabetes, she suffered with recurrent oral and genital herpes and had a hysterectomy in 1988 for CIN I and menorrhagia.

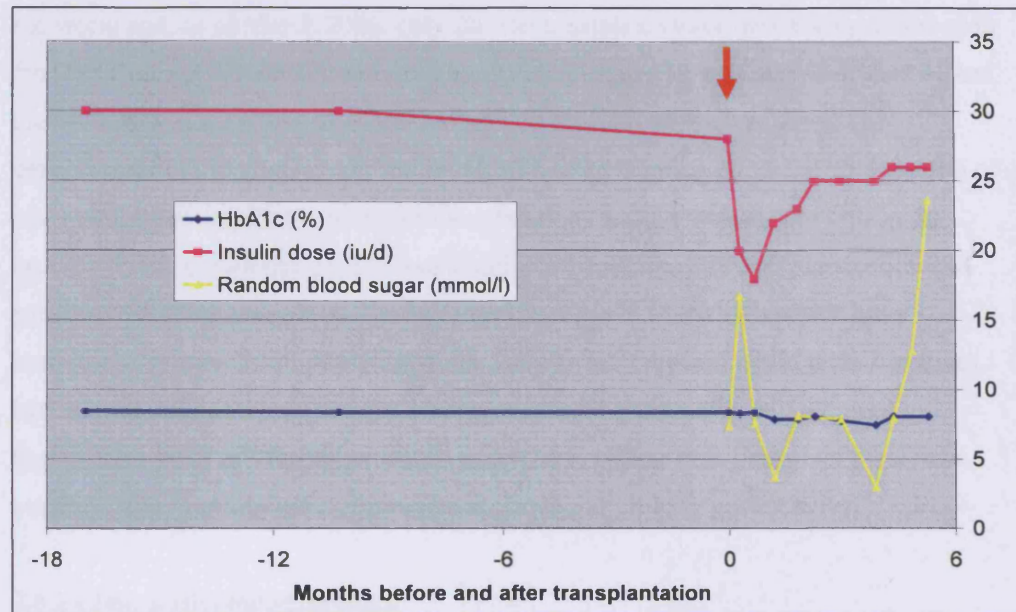
On the 24th of August 2005, she received an islet infusion of 700,000IEQ (11,900IEQ/Kg) of 97% viability. The portal vein cannulation was difficult and associated with pain. The patient also received a higher dose of heparin due to intra-cannula heparin being given with heparin in the bag with the islet transplant. These occurrences culminated in a large intra-abdominal haemorrhage of 2-3 units which was associated with profound (80/40) hypotension, in the absence of reflex tachycardia caused by her autonomic neuropathy. Her bleeding resolved spontaneously and subsequent radiological imaging did not show any evidence of hepatic bleeding. She had a 2 unit transfusion after her haemoglobin fell to 8.4g/dl and luckily, subsequent Luminex analysis has not shown any indication of sensitisation.

The third recipient also experienced epigastric pain and fullness for 10 days after the transplant which was associated with a rise in liver enzymes to an ALP of 156U/l, AST of 128U/l and ALT of 187U/l. The white blood cell count fell to $2.53 \times 10^9/l$ by 2 weeks after the transplant but has slowly risen back toward normal with no change in therapy and without stopping the prophylactic agents. Due to her history of previous oral and genital herpes and the experience with the first patient, the third recipient also received HSV prophylaxis with valacyclovir. For three months after her transplant, she did not have any evidence of genital or oral ulceration but has had one or two small oral ulcers since.

Despite the large dose of islets infused, her glycaemic control has only improved marginally and she still has episodes of hypoglycaemia, although less severe than before her transplant. Her basal C peptide remains undetectable and it is unclear whether her graft is functioning at all. She has not been sensitised by the transplant and does not have detectable islet antibodies post transplant; either by screening luminex or PRA analysis. Therefore, it is presumed that her graft was irreversibly damaged at the time of transplantation due to her episode of hypotension and poor hepatic perfusion. Some of her metabolic parameters are highlighted in figure 7.10.

Four months after her transplant, she developed some oral ulceration and has more recently developed an acneform eruption on her hands and face. Both of these occurrences are recognised side effects of Sirolimus treatment and, as of January 2006, her Sirolimus was discontinued because she had side effects of immunosuppression without the benefits of a functioning graft. The Tacrolimus therapy has been retained in order to reduce the risk of allo sensitisation and as of February 2006, she is still awaiting a further transplant.

Figure 7.10: Selected parameters from recipient 3. The graph shows values for random blood glucose, glycated haemoglobin and insulin dose per day over time before and after transplantation. The red arrow indicates the timing of her islet infusion.



7.6 Discussion

7.6.1 Introduction

Despite the successes of the Edmonton protocol, the UK has lagged behind the rest of the world and, as of March 2006, only 20 islet transplants have ever been performed in the UK. Our data has shown that islet transplantation can be established in the UK and the successes of the Edmonton protocol can be transferred to other units. The development of our programme has involved a steep learning curve for the isolation of islets which has required centre specific alterations to the protocol. The change in enzyme blend, circuit design, DNase addition, temperature control, purification and pancreas handling have all lead to a gradual increase in islet yields which have culminated in successful islet transplants. Despite our own and world wide successes, islet transplantation is still an imperfect clinical tool and work is still required to improve the yield of islets from whole pancreases, reduce post transplant β -cell loss, improve long term immunosuppression regimes and prolong graft survival.

7.6.2 Clinical islet transplantation

The first step in establishing a clinical islet transplant programme is to achieve a minimal standard of success through reduplication of the Edmonton experience. Our three patients had a full range of experience from probable primary non function through to the first single donor to recipient success in the UK. Our first two recipients achieved dramatic reductions in their insulin doses which was associated with initial sensing of hypoglycaemia and, as their insulin dose was reduced, abolition of hypoglycaemia. The life altering impact of these achievements can not be underestimated and this is reflected in a reduction of fear of hypoglycaemia scores (data not presented) and improvements in quality of life. Our first recipient has been transformed from a patient who was housebound from her diabetic complications to an unrestricted individual who is able to enjoy boundless social interaction, unfortunately to the detriment of her obvious carer patient relationship. Islet transplantation should therefore not be viewed purely as a tool for improving diabetic control but as a method for improving quality of life in patients with the severe secondary complications of their type 1 diabetes.

The post transplant outcome for our patients was variable but probably reflects the worldwide experience of replicating the Edmonton protocol. A certain level of success and failure is to be expected when transferring an established technique to another unit and comparison with other islet transplant centres is required. The interim data from the Immune Tolerance Network trial (presented by James Shapiro at the American Transplant Congress in 2004) provides a bench mark for the level of success to expect when the protocol is adopted world wide. The data for the first 36 recipients in the trial has shown that 53% of patients achieve insulin independence after one to three islet grafts and a further 19% have abolition of hypoglycaemia and improved metabolic control on reduced doses of insulin. Technical and transplant related failure rate can be expected and the trial reported a 17% primary non function rate with an additional 11% of patients withdrawing from transplantation due to side effects (Shapiro, Ricordi et al. 2004).

Our transplant protocol was modified from the original Edmonton publication and most of the changes were made to the isolation process. One important change was the use of islet culture prior to transplantation which may have affected the clinical outcomes of islet recipients. The original Edmonton protocol attempted to transplant islets as fresh as possible (Shapiro, Lakey et al. 2000) but subsequent publications suggested that a period of static culture may improve islet purity (Froud, Ricordi et al. 2005; Warnock, Meloche et al. 2005). Our protocol incorporated a period of culture in order to improve islet purity, allow detailed microbiological assessment of the preparation and enable the transplant procedure to be performed during daytime working hours. Although a period of islet culture may have resolved logistical issues related to transplantation, it is possible that this may have adversely affected the outcome in our transplant recipients and subsequent publications (King, Lock et al. 2005; Olsson and Carlsson 2005) have shown that culture prior to transplantation may negatively affect glucose resolution in experimental transplant models. Therefore, subsequent islet transplants should probably be performed as fresh as possible and only incorporate a very brief period of culture long enough for rapid assessment of the graft and organisation of transplant logistics.

7.6.3 Complications of islet transplantation

Islet transplantation is a relatively safe procedure when compared to other transplant techniques. The main procedural complications are related to bleeding and portal vein thrombosis (Shapiro, Lakey et al. 2000; Ryan, Paty et al. 2005) and a fine balance exists between thrombosis and the risk of anti-coagulant induced haemorrhage. One of our recipients suffered an intra peritoneal haemorrhage which probably contributed to the loss of her islets. This complication was possibly related to the dose of anti-coagulant she received and we have subsequently modified our protocol. The intra-peritoneal haemorrhage also raised the issue of cardiovascular disease and screening in our patient work up. All patients were initially required to have a formal stress echo cardiogram and subsequent evaluation of coronary arteries, if necessary. With the delay of stress echo appointments and the first two uncomplicated transplants, this policy was reconsidered. Our third recipient had a normal myocardial perfusion on stress testing. When she bled, her diabetic autonomic neuropathy meant she did not mount a tachycardia with her hypovolaemia and her bleeding was only diagnosed when her blood pressure dropped precipitously. This situation highlights the hazards involved with transplanting high risk patients and the necessity for full cardiovascular work up in patients who may have silent cardiac ischaemia. If this patient had had significant undiagnosed coronary vascular disease at the time of her transplant and bleed, she may have suffered a stress induced myocardial infarct which could have had significant impact on her survival and the progression of our programme. In view of this patient's complication, the policy of full cardiovascular evaluation is currently maintained prior to transplantation.

The greatest risk from islet transplantation relates to the use of long term immunosuppression and all patients should be fully informed of the risks. The main complications we encountered were related to the side effects of sirolimus. All patients developed mouth ulcers post transplant and one recipient required hospitalisation from a secondarily infected ulcer. The ulceration was so severe in one case that the first recipient was unable to eat for two to three days. Mouth ulceration is a common side effect of sirolimus use (van Gelder, ter Meulen et al. 2003) and occurs in up to 20% of kidney recipients taking 5mg of sirolimus per day (MacDonald 2001) and 89% of

patients on the Edmonton protocol (Ryan, Paty et al. 2005). There is an association between sirolimus use and the frequency of herpes infections (Kahan 2000) although not all ulcers were proven positive for herpes on culture or biopsy (MacDonald 2001). Our first patient responded well to anti-viral therapy after a positive HSV swab and subsequent patients had limited ulceration on valacyclovir prophylaxis, suggesting a possible causative link with HSV.

The other side effects encountered with sirolimus were ankle oedema, anaemia, lymphopenia, acneform rash requiring discontinuation of the drug and microalbuminuria. The microalbuminuria and proteinuria is a serious complication of sirolimus use and is worse if patients have underlying urinary protein abnormalities prior to sirolimus initiation (Senior, Paty et al. 2005). Although the urinary protein abnormalities reverse on withdrawal of the drug (Senior, Paty et al. 2005), there is an associated reduction of renal function that does not appear to be reversible.

The increasing list of side effects and the anti-angiogenic properties of sirolimus (Guba, von Breitenbuch et al. 2002) have thrown doubt over whether it is the right agent for islet transplantation. The drug was successful in achieving a steroid free immunosuppression protocol and a number of transplant centres initially moved to copy the Edmonton experience. The time has probably come to look at other immunosuppression agents and mycophenolate mofetil or campath1-H may be the solution to the side effects of sirolimus.

7.7 Conclusions

Our experience with islet transplantation has confirmed that islet transplantation can be established in the UK using the Edmonton protocol. The protocol still requires further optimization in order to successfully achieve insulin independence in a recipient from a single donor pancreas and reduce the short and long term side effects of transplantation. The long term resource issues need to be addressed and central funding is required for this clinically important technique to survive in the UK.

Chapter 8 – Discussion

8.1 Background and aims

Diabetes is a common disease which has important implications for healthcare providers around the world. The increasing incidence of both type 1 and type 2 diabetes will have a major financial and social impact on the global population (King, Aubert et al. 1998; Onkamo, Vaananen et al. 1999) which will probably be due to the effect of the secondary complications and disability caused by diabetes, rather than the direct effects of diabetes treatment (ADA 2003).

The secondary complications of diabetes are associated with poor glycaemic control and improving control can reduce their incidence (DCCT 1993). Unfortunately, achieving this goal is difficult and is associated with life threatening episodes of hypoglycaemia. Transplantation of islets, either in association with a whole pancreas or as islets alone, provides a method for achieving excellent metabolic control without the associated risk of hypoglycaemia (Robertson, Sutherland et al. 1996; Shapiro, Lakey et al. 2000). Islet transplantation is now an established treatment for type 1 diabetic patients with severe secondary complications (Shapiro, Lakey et al. 2000) but is limited by the large quantity of islets needed to induce insulin independence and the poor long term graft survival; under 10% insulin independent at five years (Ryan, Paty et al. 2005). These two interrelated issues remain the stumbling blocks of islet transplantation and their resolution should ensure a more widespread uptake of this important clinical tool.

The short and long term insulin independence rates are probably dependant on the early survival of islets within the peri- and immediate post transplant period. The high level of graft loss immediately post transplant (Biarnes, Montolio et al. 2002) is probably related to a lack of functional vasculature, the time taken to reform a new network of blood vessels (Vajkoczy, Menger et al. 1995) and the low partial pressure of oxygen achieved within the islets (Carlsson, Palm et al. 2002). If the revascularisation process could be targeted in order to accelerate the revascularisation process and improve oxygen delivery

to the islet, it may be possible to reduce the immediate loss of islets post transplantation and improve long term outcomes.

8.2 The revascularisation of intra-portal transplanted islets

8.2.1 Introduction

Islet revascularisation is an essential step in the engraftment of transplanted islets. During this important process, a high percentage of transplanted islets are lost and successful revascularisation probably holds the key to initial and long term outcomes. A better understanding of revascularisation is required to improve engraftment and hopefully improve clinical outcomes so that more patients can be transplanted with the small number of donated pancreases available.

8.2.2 Models of transplanted islet revascularisation

The investigation of transplanted islet revascularisation depends on a good model of islet transplantation that provides both parallel conditions to clinical methods and allows the evaluation of scientifically important parameters. The different models used have their own individual strengths and weaknesses which guide the investigator to choose the model most relevant to their individual research.

Kidney subcapsular methods of islet transplantation provide a well vascularised site of engraftment for islets with easy retrieval of the entire graft. The kidney subcapsular model has proved popular for the assessment of mRNA (Vasir, Jonas et al. 2001), oxygen tensions (Carlsson, Palm et al. 2000) and vascular density (Carlsson, Palm et al. 2002) within transplanted islets and has been used for the assessment of human islets transplanted into immunodeficient mice (Carlsson, Palm et al. 2002). Dorsal skin fold models of islet transplantation provide a further site for transplantation which allows easy retrieval of the entire graft and also presents a useful tool for direct in-vivo visualisation of graft revascularisation in real time (Vajkoczy and Menger 1995).

The limitation of these transplant models lies in the lack of clinical relevance, as most clinical islet transplants are performed intraportally, and the clumping of cells after transplantation rather than wide dispersal throughout a vascularised organ. The clumping of transplanted islets increases the diffusion distances for molecules and may alter the

conditions relevant to islet engraftment. Although intra-portal transplant models obviate these problems, they are limited by the lack of full retrieval of the graft and the wide dispersal of islets which makes histological analysis of transplanted islets difficult. The right branch transplant model, as developed within our laboratory (Juszczak M 2003), has improved the density and histological recovery of transplanted islets within liver sections and we have been able to visualise an average of 4.5 islets per liver section. This model was further extended by the use of dual BS-1 and insulin staining to allow both qualitative and quantitative analysis of islet vascular architecture and track the revascularisation of transplanted islets within the liver. Therefore, the current model enables better sampling and analysis of islets within the more clinically relevant transplant intra-portal transplant site.

8.2.3 Revascularisation of intra-portal transplanted islets

The complex vascular network of native islets is disrupted during isolation from the whole pancreas and islets reform a new blood supply from the surrounding tissue (Andersson, Korsgren et al. 1989; Vajkoczy and Menger 1995). Passenger endothelium within the islet starts to disappear during the first few days post transplant (Furuya, Kimura et al. 2003) and new vessels invade the islet to form a new network of vessels. Revascularization is initiated by capillary sprouting between day 2 to 4, which results in fusion of vascular channels by day 6 post transplantation and eventual formation of a glomerulus like network of blood vessels (Vajkoczy, Menger et al. 1995). Complete revascularization of islets has been estimated to occur by day 10-11 post transplantation, irrespective of whether the islets are transplanted into the liver (Griffith, Scharp et al. 1977) or the dorsal skin fold (Vajkoczy, Menger et al. 1995).

The initial stage of islet revascularisation is a reduction in islet endothelium which is followed by the formation of a new vascular network. The initial reduction of endothelial staining has been observed by a number of authors (Griffith, Scharp et al. 1977; Furuya, Kimura et al. 2003; Nyqvist, Kohler et al. 2005) and is demonstrated here by the fall in percentage of islet comprised of endothelium and reduction in vascular density over the first few days post transplant. The revascularisation of transplanted

islets was initially shown to arise from cells of host origin (Vajkoczy and Menger 1995) and it was assumed that donor endothelium, within islets at the time of transplantation, was lost early after transplantation and did not play any role in the new vascular network. Subsequent work has shown that isolated islets suspended in fibrin gel can form endothelial sprouts from the islet core without the need for host tissue and suggests that donor islet endothelium does not necessarily disappear after isolation (Linn, Schneider et al. 2003). The same publication described the use of a transgenic mouse with a Tie2-LacZ reporter construct which enabled visualization of donor endothelium in islet grafts up to 3 weeks after transplantation (Linn, Schneider et al. 2003) and lead the authors to conclude that the endothelium was not lost but may have dedifferentiated after isolation and subsequently redifferentiated to participate in the revascularization process. This theory was further substantiated by the use of a Tie-2 gfp (green fluorescent protein) mouse which is characterized by endothelial expression of gfp. Cultured islets from this animal show a reduction in islet endothelium, as indicated by the loss of CD31 expression, over time but culture encouraged the appearance of a population of CD31 negative/gfp positive cells, suggesting that the islet endothelium may have dedifferentiated and lost its CD31 cell surface marker (Nyqvist, Kohler et al. 2005). The islets from this transgenic mouse were then transplanted into athymic nude mice and were found to have islet endothelium expressing gfp after revascularization, suggesting that the donor endothelium survives after transplantation to participate in the new islet vascular architecture (Nyqvist, Kohler et al. 2005). These experiments imply that islet endothelium may dedifferentiate during the early stages of engraftment and lose its cell surface markers before redifferentiating during revascularization, in order to contribute to the new islet vasculature. Therefore, the early reduction in BS-1 staining after transplantation may not represent loss of endothelium but may be due to the loss of endothelial carbohydrates specific for the binding of BS-1, caused by endothelial dedifferentiation. Unfortunately, the Y chromosome FISH was not suitably robust to use for analysis of islet endothelial origin and it is not possible to speculate whether these cells subsequently redifferentiate to take part in revascularisation.

The assessment of islet revascularisation, presented here, agrees with previous descriptions but suggests that islets are revascularised at an earlier time point and may already be revascularised as early as day 5 post transplantation. The period between day five and fourteen are probably associated with a consolidation of vascular architecture, as seen by the rise in vascular density and branching index, and is most likely to represent a period of formation of the multibranching glomerular network.

The changes in vascular architecture continue up to 30 days post transplant and suggest that vascular remodelling is still ongoing at this time point. The experiments were not continued beyond 30 days post transplant and it is therefore impossible to say whether further remodeling occurs after this time point. If the fresh group from the 350 islet desferrioxamine treatment experiments are taken into account, the results would suggest that although the size of islets are not significantly different, the vascular density and branching index of freshly transplanted islets after 11 weeks is significantly less than at day 30; grid density after 11 weeks- 2328 vessels per mm^2 vs. 3172 vessels per mm^2 at 30 days ($p>0.01$) and 0.76 vs. 0.87($p>0.01$) respectively. Interestingly, when the fresh islets from the 350 islet experiments are compared to native islets, the vascular density is higher in the transplanted islets but the branching index is the same; grid density after 11 weeks- 2328 vessels per mm^2 vs. 1826 vessels per mm^2 in native islets ($p>0.01$) and 0.76 vs. 0.77 ($p=0.94$) respectively. These comparisons would suggest that further remodeling does occur with time and the vascular architecture changes back toward that found in native islets.

One note of caution for this extrapolation is that the dose of islets in the control culture group from the desferrioxamine treatment experiments was much smaller than that used for the revascularisation experiments; 350 islets in the DFO experiments compared with an average of 752 islets in the revascularization experiments. This factor may have affected engraftment and revascularization, as it has been proven that engraftment may be effected by early glycaemic control (Biarnes, Montolio et al. 2002), although other authors suggest have suggested that early glycaemic control does not effect vascular density (Mattsson, Jansson et al. 2003).

8.2.4 Methods for assessing vascular density

The experimental comparison of islet engraftment requires a quantitative method for analysing vascular architecture between different experimental groups. Vascular density is frequently used to assess the engraftment of islets after transplantation and higher values have been suggested to denote improved islet engraftment. The measurement of this parameter is not standardized and the vascular density of transplanted islets has been assessed using a number of different methods. The two main methods either assess the total length of vessels per islet area (Vajkoczy and Menger 1995; Beger, Cirulli et al. 1998) or the number of vessels per area. These two methods produce different numerical results which can not be directly compared but within each method they provide comparative data to explore the effect of different experimental conditions.

The question remains whether one or other technique is more reproducible between different groups and which is less likely to be subject to operator bias. This situation has been investigated within malignant tumors (Weidner, Semple et al. 1991; Goddard, Sutton et al. 2001; Vermeulen, Gasparini et al. 2002; Sharma, Sharma et al. 2005) and the consensus amongst pathologists suggests that most use a modified system based on the original description by Weidner (Weidner, Semple et al. 1991). Weidner described a method for identifying a “hotspot” (area of increased vascular density within a tumour) and counting the number of vessels within a 200x field which were stained for factor VIII. The microvascular density was subsequently found to have positive correlation to metastatic spread of breast tumours (Weidner, Semple et al. 1991). A modification of this system, using the islet as the hotspot area, has been used here and by other authors (Carlsson and Mattsson 1993; Mattsson, Jansson et al. 2002). The problem with this system relates to observer bias when counting vessels and whether two adjacent stained structures represent the reflection of a single vessel or two separate vessels. One suggested method for overcoming this bias is to use a grid, where the density is calculated by counting grid hits (stained vessels crossing a part of the grid) per area. This system has been suggested to reduce bias and has been positively correlated with outcome in breast cancer (Fox, Leek et al. 1995). A modification of this system was also

used here for the assessment of vascular density and gave a different perspective to the examination of islets vascular architecture.

The vessel count method indicates the number of vessel branches per area but non branching vessels score low using this system. The grid method will calculate a higher score for the same islet with non branching vessels but an islet with small branching vessels, as seen in a glomerular like network, will score more equally with the two systems. This observation allowed the analysis of the islet vascular network by comparing the two methods and calculating the branching index, with higher values suggesting a greater degree of branching. The result generated by the branching index shows a good correlation with the gross morphology of islet vascular architecture and changes in an expected and progressive way during engraftment. This would suggest that the branching index, in addition to vascular density, provides useful information for assessing islet vascular architecture during engraftment.

The current evaluation of transplanted islet revascularisation relies on the assessment of gross islet morphology and vascular density but it is unclear whether these techniques are the most appropriate methods to assess revascularization. The field of islet revascularization could learn a great deal from the oncology pathologists who, like islet researchers, study the vascularisation of metastatic tissue. The question remains whether the state of differentiation or composition may be a better indication of vasculogenesis than purely measuring the number of blood vessels per area (Vermeulen, Gasparini et al. 2002). Therefore, the analysis of different cell surface markers or surrounding supportive cells may give a better indication to how well an islet has revascularised rather than purely assessing vascular density.

8.2.5 Vascular density in native and transplanted islets

Islet engraftment is a complex process and requires the formation of a new vascular network. When the islets have fully engrafted, the new vascular supply is poor when compared to that found in native islets and oxygen tensions within transplanted islets are lower than those found in native islets (Carlsson, Palm et al. 2000; Carlsson, Palm et al. 2002). In addition, the vascular density of transplanted islets has been reported to be lower than in native islets (Carlsson, Palm et al. 2002; Mattsson, Jansson et al. 2002; Mattsson, Jansson et al. 2003), suggesting poor revascularization of transplanted islets. These observations are in contrast to the data presented here which suggests that although the vascular density falls immediately after transplantation, it returns to normal and subsequently overshoots to a level above that found in native islets.

The numerical and gross observation of intra portal transplanted islets presented here does not suggest that islet vascular density is reduced after revascularization has occurred. Before considering the reasons for this discrepancy with reported literature, the methodology should be examined to exclude errors in assessment. Firstly, the staining of transplanted islet endothelium is difficult (Mattsson, Carlsson et al. 2002) and any differences in staining between transplanted and native islet endothelium could lead to a discrepancy. This cause for error would seem unlikely as endothelial staining within native islets was bright and there were no obvious unstained areas which could have correlated with endothelium. In addition, the numerical data presented for native islets (1358 vessels per mm² in native islets) is similar to the values from other authors using a similar methodology (~1300 vessels per mm²) (Mattsson, Jansson et al. 2002) and it is unlikely that either the staining or density assessment methodology is the cause of the discrepancy between native and revascularised transplanted islets.

If the methodology for assessing vascular density is not the cause for the difference, why is there a discrepancy? One possible explanation for the discrepancy is the model of revascularization used to investigate islet transplantation. Most of the studies have been performed using kidney subcapsular models of islet transplantation (Carlsson, Palm et al. 2002; Mattsson, Jansson et al. 2003). This model involves clumping the entire graft

together under the kidney capsule and does not distribute the tissue widely throughout the organ. It is possible that this clumping not only leads to increased diffusion distances for important molecules but also reduces contact with the host tissue and local growth factors which are vital for new vessel formation. This situation is highlighted by the connective tissue seen around islets implanted beneath the kidney capsule which is not seen when islets are transplanted into the liver (Mattsson, Jansson et al. 2002). The contact of cells with endothelium is important for angiogenesis and in a model of cancer metastases, it has been shown that the contact of tumor cells with endothelium is important for metastasis proliferation, migration and early vessel formation (Barrett, Mangold et al. 2005). In addition, endothelial I-CAM 2 has been shown to regulate angiogenesis (Huang, Mason et al. 2005) and it may be that islet-endothelial interaction is important for engraftment and subsequent revascularization. Islet endothelium interaction is not present in islets that have been transplanted under the kidney capsule and may give a false indication of revascularization when compared to islets transplanted into the liver.

The reduced contact of endothelium with transplanted tissue does not explain the lower vascular density found in intra portal transplanted mouse islets, compared to native mouse islets, in the paper by Mattson and his colleagues (Mattsson, Jansson et al. 2002). The reason for this is unclear as dual insulin and BS-1 immunohistochemistry staining was used on consecutive sections, in order to localize islets within the liver. Unfortunately, the example shown in the paper does not show any endothelial staining within the intra-portal transplanted islet and it is difficult to judge the pattern of revascularization in their model. The other important factor may have been the use of 3-4 days islet culture prior to transplantation, which has subsequently been shown to reduce vascular density after transplantation when compared to non cultured islets (Olsson and Carlsson 2005).

The question of whether intraportally transplanted islets do have a lower vascular density requires further clarification with additional studies. If the observations here are reproducible, the next issue is why the vascular density of transplanted islets is higher

than that found in native islets. This probably relies on how islets revascularise and how endothelium knows where to proliferate before forming new vessels. The imaging of islets early during revascularization suggested there may be spaces left between the beta cell mass by endothelium which had regressed or dedifferentiated. As the islets revascularise, it may be that new endothelium grows back along the old vascular channels, possibly on remnant basement membranes from the native vasculature. If this were the case, the number of vessels within the islet would remain the same but the area of the islet would be reduced due to loss of beta cell mass during engraftment, therefore leading to an increase in measured vascular density in transplanted islets when compared to native islets. However, this does not explain the doubling of vascular density from 1358 vessels per mm^2 in native islets to 2715 vessels per mm^2 in day 30 islets, as the loss in islet area between these time points is only 18%. Even when the differences between three dimensional structures and two dimensional sampling are taken into account, this theory does not explain the increase in vascular density.

All measured indices of islet vascular anatomy were different between native and day 30 transplanted islets which suggest that transplanted islet vascular architecture is completely different from native islet architecture. In this case, it is probable that endothelium does not regrow down the predetermined channels left when the native islet endothelium regressed but the intense hypoxic signal within islets causes extensive random endothelial ingression into the islet. This intense revascularisation may account for the increased percentage islet endothelium and vascular density seen within transplanted islets but the size of islets and locality to blood supply would be unlikely to induce such an intense hypoxic response. If a parallel with malignant tumour growth is examined, the average islet of 150 μm diameter is well below the 1mm diameter of maximal avascular tumour growth (Gimbrone, Leapman et al. 1972) and the 100 μm diffusible distance for oxygen (Helmlinger, Yuan et al. 1997). Nevertheless, small tumours of 100-200 μm still induce angiogenesis from host vasculature when implanted into well vascularised sites, with vascular densities similar to that of the surrounding tissue (Vajkoczy, Farhadi et al. 2002). Interestingly, when Vajkoczy used the same nude mouse model for studying the revascularisation of islets (Vajkoczy, Menger et al. 1995),

the functional capillary density within islets ($650\text{--}700\text{cm}^{-1}$) was twice that found on the striated muscle (300cm^{-1}) in the tumour revascularisation paper suggesting that islets may have a greater angiogenic capacity when implanted into foreign tissues. Unfortunately, the islet transplant paper (Vajkoczy, Menger et al. 1995) did not describe any analysis of or comparison to the functional capillary density of the underlying muscle bed and it is impossible to fully validate this conclusion.

The environment into which tumours are implanted has an influence on their growth and outcome (Radinsky and Ellis 1996) with different implantation sites directly affecting vascular architecture (Fukumura, Yuan et al. 1997; Bernsen, Rijken et al. 1999). The vascular architecture of the liver is complex and blood from portal venules and hepatic arterioles drains through the hepatic sinusoids into the hepatic venules (Oda, Yokomori et al. 2003). The factors controlling and maintaining the development of this complex vascular architecture have not been fully elucidated but probably require the balance of local growth factors such as VEGF and pigment epithelium derived factor (Gouysse, Couvelard et al. 2002; Sawant, Aparicio et al. 2004). It is possible that the liver specific balance of these local growth factors may also regulate the vascular architecture of non malignant implanted tissue and may explain why the islet architecture changes after transplantation into the liver.

The liver is highly vascular and receives 30% of cardiac output from its dual blood supply but the oxygen tension within the main portal vein of air breathing rats is low ($\sim 38\text{mmHg}$) (Hughes, Yang et al. 2004) and subsequent hepatic parenchymal oxygen levels have been reported to be even lower at 4 - 16 mmHg (Mucke, Richter et al. 2000; Carlsson, Palm et al. 2001). Therefore, the oxygen tension within the normal liver parenchyma is close to the 10-15mmHg necessary for 50% maximal induction of HIF-1 found in HeLa cells (Jiang, Semenza et al. 1996) and the 9mmHg necessary for the 50% maximal induction of VEGF, measured within the same cell line (Chiarotto and Hill 1999). The low oxygen tension within the liver (4mmHg) contrasts with values of 13mmHg and 30mmHg reported in the same paper for the renal cortex and exocrine pancreas, respectively (Carlsson, Palm et al. 2001). Although the values for tissue

oxygen tension reported in the paper by Carlsson are lower than those reported by other authors (Schurek, Jost et al. 1990; Mucke, Richter et al. 2000), the important comparison is the constant half to two thirds lower oxygen concentration in the liver compared to the kidney. The lower oxygen concentrations within the liver may suggest why there is a greater hypoxic drive to revascularisation when islets are transplanted into the liver when compared to the more commonly used renal subcapsular model.

8.2.6 Conclusions

Transplanted islet revascularisation is a complex process which occurs in a stepwise fashion from initial reduction in endothelium through to reformation of a new and increased vascular network. This process is quantifiable and the novel methods described here can be used for the assessment and comparison of islet revascularisation under different experimental conditions. The final vascular density reported here is higher than that found in native islets and this contrasts to previous reports. This difference in vascular density can probably be explained by the differences in models and manipulation of islets prior to transplantation. Although a higher vascular density in transplanted islets is unusual, this may be explained by the implantation of islet tissue into a different and more hypoxic tissue than its native environment.

8.3 Desferrioxamine treatment of islets

8.3.1 Effect of desferrioxamine treatment on isolated islets

Desferrioxamine has been successfully used for the treatment of transfusion dependent iron overload since the early 1960s (Hershko, Konijn et al. 1998) with proven reduction in patient mortality (Gabutti and Borgna-Pignatti 1994). Although DFO is widely used for iron overload, its hydrophilic nature results in poor penetration of cell membranes (Zanninelli, Glickstein et al. 1997). This characteristic of DFO not only requires higher concentrations and longer exposure of the cells to the drug, when compared to other lipophilic iron chelators, but DFO also requires a longer time to exit the cell (Richardson, Ponka et al. 1994) and chelated complexes of DFO are still detectable in the cell after 20 hours post exposure (Cooper, Lynagh et al. 1996). On the other hand, this property means that DFO does not enter the mitochondria and does not affect mitochondrial function (Richardson, Mouralian et al. 2001).

The properties of desferrioxamine suggested that it would be a good compound for exploring the effect of iron depletion in islets. The treatment of islets was carried out in overnight culture and the length of time required for DFO to enter the cells was not an issue. Conversely, the hydrophilic nature of DFO and slow cellular washout was an important property and allowed the sustained effect of DFO after the islets were transferred into an iron containing environment. The higher concentrations of DFO produced a greater effect on VEGF expression and enabled a more prolonged expression than seen in lower doses but did not affect important cellular functions; probably as a result of DFO's inability to enter the mitochondria.

The treatment of islets with DFO produced a dose dependent increase in the expression of VEGF but did not increase the expression of the other growth factors important for the engraftment of islets (Vasir, Reitz et al. 2000; Menger, Yamauchi et al. 2001). This finding was disappointing as earlier publications in islets had suggested that hypoxia up regulated HGF (Vasir, Reitz et al. 2000) and that HGF was an important factor for the engraftment of islets (Nakano, Yasunami et al. 2000; Garcia-Ocana, Takane et al. 2003;

Lopez-Talavera, Garcia-Ocana et al. 2004). In addition to the down regulation of HGF, desferrioxamine treatment and overnight culture was associated with a reduction in the expression of bFGF and Ang1. These two growth factors are probably important for islet engraftment as bFGF has an important angiogenic capacity (Okada-Ban, Thiery et al. 2000) and Ang1 is known to stabilise endothelium (Sato, Tozawa et al. 1995; Thurston, Rudge et al. 2000). Therefore, the reduced expression of these growth factors associated with culture may lead to a reduced angiogenic capacity and regression of donor endothelium within cultured islets. If so, cultured islets would be expected to engraft worse than fresh islets and this change in growth factor expression may explain the poorer outcome in cultured islets found by Olsson and colleagues (Olsson and Carlsson 2005).

Despite the negative changes in growth factor expression, desferrioxamine treatment up regulated the expression of VEGF and increased VEGF expression has been shown to improve engraftment and clinical outcomes in experimental islet transplantation (Stagner, Mokshagundam et al. 2004; Zhang, Richter et al. 2004; Lai, Schneider et al. 2005). In addition, the treatment of islets with desferrioxamine did not reduce viability or function of isolated islets and may enhance the glucose stimulated insulin release. These important observations suggested that desferrioxamine treatment of islets was a promising technique for improving transplanted islet engraftment and should improve the outcomes after transplantation. The next step was to see whether the in-vitro data could be translated into improved outcomes in a marginal islet transplant model, using both clinical and histological endpoints to examine the effect of DFO treatment.

8.3.2 Effect of desferrioxamine on transplanted islets

The hypothesis for improved outcomes is that DFO up regulates the expression of VEGF prior to transplantation and reduces the lag time to peak production of this important growth factor after transplantation. It was hoped that the earlier peak of VEGF expression would reduce the time necessary for revascularisation, leading to a reduction in hypoxia and therefore increased survival of beta cell mass. In addition, hypoxia preconditioning with desferrioxamine should reduce the susceptibility to hypoxia induced cell death and when combined with improvements in revascularisation, lead to better early and long term transplant outcome. Unfortunately, the clinical outcomes did not shown any improvement in either the reversal of diabetes or the rate of glucose reduction post transplantation. The cause for this failure is unclear and the issues pertaining to the experimental model have been considered in chapter 6. The following section will consider whether the hypothesis may have been flawed.

8.3.2a Action of desferrioxamine and availability of iron

The premise for treating islets with DFO prior to transplantation is to induce prolonged over expression of VEGF and precondition islets to withstand hypoxia induced cell death. The in-vitro experiments confirmed a prolonged over expression of VEGF with DFO but it is plausible that these experiments did not sufficiently mimic the real life situation. The in-vitro desferrioxamine experiments were carried out in CMRL (iron-free) medium supplemented with foetal calf serum (FCS), with subsequent transfer into M199 (iron-containing) medium supplemented with FCS. It was hoped that these experimental conditions would simulate the transfer of DFO treated islets into the iron concentrations found in serum after transplantation.

Although M199 contains similar concentrations of free iron to normal plasma and FCS contains ferritin, it is possible that these culture conditions do not mimic the availability of iron after transplantation into the liver. Therefore, the islets may have remained iron depleted after transfer to M199 and the prolonged secretion of VEGF may have been related to poor iron repletion rather than any prolonged effect of DFO treatment. When the islets were transplanted into the rat recipients they would have been embolised into

liver and bathed in surrounding blood. Both of these media contain high concentrations of free iron and islets may have become iron replete shortly after transplantation. If this was the case, the islets would not have over expressed VEGF for a prolonged period and would not have augmented engraftment.

8.3.2b Administration of desferrioxamine

The preconditioning of islets with DFO was expected to reduce the hypoxia related cell death associated with transplantation. Islets are known to be hypoxic after transplantation (Carlsson and Mattsson 1993) but it is unclear whether this is the greatest hypoxic insult the islets suffer. The isolation of pancreatic islets is a prolonged procedure (4 hours in rodents and 10 hours in humans) which is preceded by the warm and cold ischaemic phases of pancreas retrieval. Although the percentage of islet apoptosis and necrosis presented here is low, other authors have published apoptosis rates of 20-50% after isolation (Cattan, Berney et al. 2001; Ichii, Inverardi et al. 2005), suggesting that the hypoxic insult may occur during pancreas retrieval and isolation and before transplantation. If so, the point at which islets were treated with DFO would be too late to protect islets from maximal hypoxic insult and a better time to administer the DFO may be at the time of retrieval. Therefore, islets would be preconditioned before the insult of pancreas retrieval and islet isolation. Further experiments are probably required to explore this hypothesis.

8.3.2c The importance of VEGF to islet engraftment

Vascular endothelial growth factor is known to be important for angiogenesis (Ferrara 2004) and is expressed during the early stages of islet revascularisation (Vasir, Jonas et al. 2001). The up regulation of VEGF in experimental models has been shown to improve islet engraftment (Stagner, Mokshagundam et al. 2004; Zhang, Richter et al. 2004; Lai, Schneider et al. 2005) but it remains to be proven whether VEGF is truly pivotal in the revascularisation of islets. Two further studies have provided some insight into this issue and have looked at VEGF expression in cultured islets and the effect of VEGF receptor blockade on islet engraftment.

Vascular endothelial growth factor expression is known to be up regulated in islets during culture (Gorden, Mandriota et al. 1997; Vasir, Aiello et al. 1998; Vasir, Jonas et al. 2001; Olsson and Carlsson 2005) but when cultured islets are transplanted, their outcomes are worse than fresh islets which have a lower VEGF expression (Olsson and Carlsson 2005). In addition, islets transplanted into recipients treated with VEGF receptor blockade have similar outcome and vascular density when compared to islets transplanted into control recipients, suggesting that signalling through the VEGF receptor plays less of a role in islet engraftment. These observations suggest that VEGF and its downstream effects are not a prerequisite for islet engraftment and other factors probably play an equally important role. Therefore, the use of desferrioxamine to up regulate VEGF alone may not be the answer to improving islet engraftment.

8.3.3 Conclusions

The treatment of islets with DFO has been shown to cause a sustained increase in the expression of VEGF without adversely affecting viability and may improve islet glucose responsiveness. Unfortunately, DFO treatment did not improve the change in weight or reversal of diabetes post transplantation but has improved some of the histological parameters of islet engraftment and may reverse some of the adverse effects of islet culture. Further experiments are required to examine whether the timing or route of administration of desferrioxamine may improve islet engraftment.

8.4 Clinical islet transplantation

8.4.1 Introduction

Clinical islet transplantation has come a long way since the first recorded non vascularised pancreas transplants in 1893. Despite the initial enthusiasm for non vascularised pancreas transplantation, the progress in this area of medicine has been slow when compared to the transplantation of other organs. Isolated islet transplantation remained unproven as a clinical technique for many years and it was not until the experience of Najarian and his colleagues in 1977 that islet transplantation was shown to be of any clinical benefit (Najarian, Sutherland et al. 1977). The subsequent advances in islet isolation from the whole pancreas (Ricordi, Lacy et al. 1988) improved transplant outcomes but insulin independence rates remained poor and it was not until the publication of the Edmonton protocol that islet transplantation was realised as a valuable treatment option for patients with type 1 diabetes (Shapiro, Lakey et al. 2000).

8.4.2 Islet transplantation in the UK

Our data has shown that islet transplantation can be established in the UK and the successes of the Edmonton protocol can be transferred to other units. The success of the programme will hopefully be continued for the transplantation of further recipients but, as with all programmes, will be limited by the supply of pancreases and funding for staff costs and consumables. The number of pancreases for isolation has already been addressed by the Diabetes UK Expolink but the additional expense of pancreas retrieval (i.e. UW and duodenal stapler) has not been covered. This situation is further compounded by the lack of funding for isolation and transplant costs which are currently part funded by Diabetes UK. Unfortunately, this funding will only cover the next 7 transplant recipients and further funding from governmental bodies, such as NSCAG, is currently being sought. If this funding is not forthcoming, the future of islet transplantation in the UK will remain uncertain.

8.4.3 The place of islet transplantation in management of diabetic patients

If islet transplantation is to be endorsed and funded by the NHS, it should be cost effective and provide a valid and necessary treatment option for patients with diabetes. At present, islets are transplanted for hypoglycaemic unawareness, severe progressive secondary complications despite best management and improving the metabolic control of patients who are already immunosuppressed for another solid organ transplant. If islet transplantation were not available, the current options would be to remain on insulin therapy or receive a whole pancreas transplant. The benefit of islet transplantation over insulin therapy is the abolition of hypoglycaemia and improved metabolic control (Shapiro, Lakey et al. 2000), although not all authors have shown significantly better control over best medical therapy in short term studies (Warnock, Meloche et al. 2005). The risk of transplantation is mainly from long term immunosuppression and when pancreas transplantation is considered, the morbidity and mortality associated with a major abdominal operation. The benefit of pancreas transplantation over islet transplantation is long term immaculate diabetic control which islet transplantation can not currently provide. The question still remains where islet transplantation should be considered in the treatment of diabetic patients.

The first treatment choice for type 1 diabetic patients is insulin which should be delivered under best care guidelines (DCCT 1993; Warnock, Meloche et al. 2005). Patients should only be considered for transplantation when best care has failed and all other treatments have been exhausted, unless they are already receiving immunosuppression for another organ. Under these circumstances, patients should be considered for either islet or whole organ transplantation.

Patients who require beta cell replacement alone are generally quite rare (0.5 – 1% of type 1 diabetics) and indications are usually restricted to severe hypoglycaemic unawareness or severe progressive secondary complications. The risk to benefit ratio of pancreas transplant alone is marginal, if not against whole organ transplantation (Gruessner, Sutherland et al. 2004). In this small group of patients, islet transplantation can stop episodes of hypoglycaemia and improve diabetic control and is probably the

best alternative. If the indication for beta cell replacement is the complications of neuropathy, pancreas transplantation is probably more advisable due to the need for long term immaculate control for stabilisation and possible reversal of neuropathy (Navarro, Kennedy et al. 1996; Allen, Al-Harbi et al. 1997; Navarro, Sutherland et al. 1997).

Type 1 diabetic patients who already have another solid organ transplant or are receiving a transplant already have the risk of immunosuppression and should be considered for β -cell replacement. Most of the literature relating to this group of patients arises from renal transplantation, where diabetes is the leading cause of end stage renal disease. In patients who are awaiting a renal transplant there is a significant long term mortality benefit to undertaking a combined kidney/pancreas transplant when compared to wait listed patients (La Rocca, Fiorina et al. 2000; Gruessner, Sutherland et al. 2004) or cadaveric kidney transplant alone (La Rocca, Fiorina et al. 2000; Reddy, Stablein et al. 2003).

When cadaveric kidney alone is compared to simultaneous pancreas kidney transplantation (SPK) there is little difference in initial survival but after 18 months to two years, the survival curves diverge and SPK patients do better in the long term (La Rocca, Fiorina et al. 2000; Reddy, Stablein et al. 2003). This benefit is probably due to improved glycaemic control which is reflected in lower rates of cardiovascular death (La Rocca, Fiorina et al. 2000). The transplantation of a kidney from a living donor initially improves survival over kidney alone or SPK but with time, the SPK and live donor survival curves start to converge and meet after about seven years (Reddy, Stablein et al. 2003). This pattern of survival is probably due to the high initial mortality associated with SPK but reduced mortality from cardiovascular disease in the long term, due to immaculate glycaemic control.

The patient and graft survival rates for SPK are continually improving (IPTR data) and all suitable type 1 patients with end stage renal failure should be considered for SPK transplantation unless a good live donor is available, where this option may be preferable. A further treatment for patients with end stage renal disease secondary to type 1 diabetes would be a live donor transplant, to benefit from a good kidney and low early mortality, followed by an islet transplant which would allow immaculate diabetic

control and probable reduction in cardiovascular death. At present it is unclear how this option would fit into treatment strategies for type 1 diabetes patients with renal disease and whether this may be the optimal therapy.

Type 1 diabetic patients who already have a functioning renal allograft can benefit from the improved metabolic control associated with transplantation of beta cell mass. The alternatives here are islet after kidney (IAK) or pancreas after kidney (PAK). This is a difficult group to draw a definitive conclusion about because the benefit of PAK is probably equivocal (Gruessner, Sutherland et al. 2004) and IAK may have a slightly higher morbidity than islet alone transplantation (Berney, Bucher et al. 2004). The benefit of IAK would be the lower procedural mortality and long term improvement in glycaemic control (Berney, Bucher et al. 2004; Gonzalez Molina, Alonso et al. 2005) but, if patients survive the post operative period with a functioning pancreas, the outcome is likely to be better long term.

Cardiovascular disease is a common secondary complication of diabetes and some patients are deemed not suitable for whole organ transplantation due to cardiovascular risk. The cardiovascular risk associated with islet transplantation is less than whole organ transplantation and a number of these patients with lesser degrees of cardiovascular disease may be suitable for islet transplantation.

In conclusion, islet transplantation is a valuable treatment modality that should be considered along side whole organ transplantation. It is unlikely that islet transplantation will be able to offer the long term glycaemic control and insulin independence of whole pancreas transplantation but there are some circumstances where islet transplantation is preferable.

8.4.5 The requirement for insulin independence

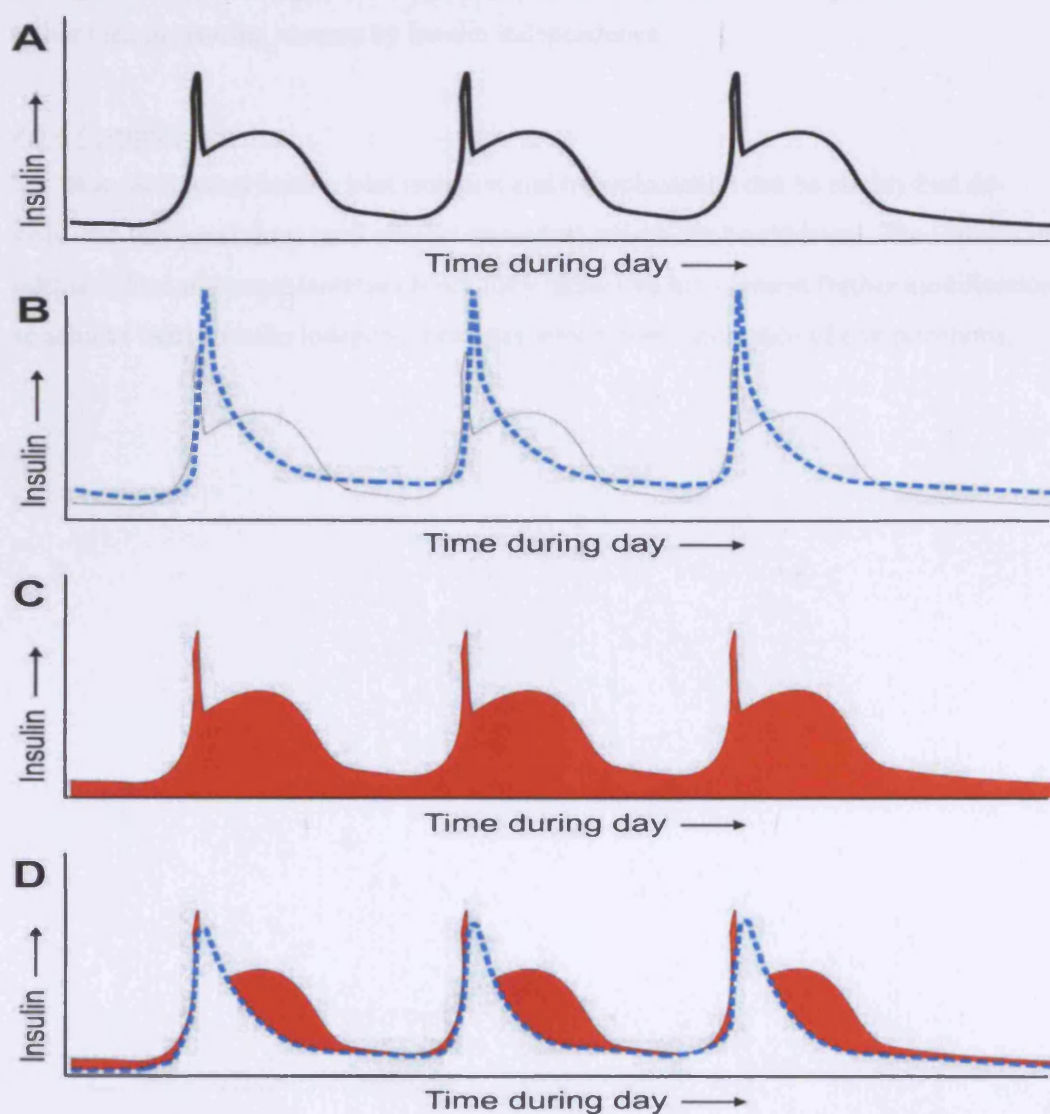
The Edmonton protocol achieved initial insulin independence rates that were comparable to whole organ pancreas transplantation (Shapiro, Lakey et al. 2000; Ryan,

Lakey et al. 2002) but with time, there has been a rapid decline in insulin independence (Ryan, Paty et al. 2005) and pancreas transplantation is currently a better modality for long term insulin independence (IPTR registry, Minneapolis). Despite the poor insulin independence rates achieved, islet transplantation abolishes hypoglycaemia (Shapiro, Lakey et al. 2000) and improves diabetic control (Ryan, Lakey et al. 2002; Ryan, Paty et al. 2005). Therefore, when considered within the initial indications of treating hypoglycaemic unawareness and improving metabolic control, islet transplantation achieves its goals successfully. This leads to the question of whether insulin independence should be the overall goal of islet transplantation. Even with the first transplant, and before insulin independence is achieved, metabolic control is improved (Shapiro, Lakey et al. 2000), hypoglycaemia is abolished (Shapiro, Lakey et al. 2000) and patients often regain a sensation of low blood glucose. The poor long term insulin independence rate remains the perceived problem of islet transplantation but we should consider whether insulin independence is the best end point to aim for or whether normalised metabolic control is a better target.

Before considering the reasons for changing targets, we should explore the cause for the high rate of attrition of insulin independence. The human pancreas contains roughly one million islets and islet isolation with collagenase digestion only yields about half to one third of the total islet mass (Lakey, Warnock et al. 1996; Shapiro, Lakey et al. 2000). After islets have been transplanted, only about 40% of the transplanted mass survives to engraft (Biarnes, Montolio et al. 2002). This poor rate of islet isolation and survival means that after one transplant, the recipient has only received between 10-30% of the normal human islet mass. The transplanted mass is often insufficient to cover the total insulin requirements of the recipient and therefore a second transplant is often needed. After the second transplant, the engrafted mass of islets is usually sufficient to cover the recipient's insulin requirements but will only just meet these requirements under a great deal of metabolic stress. This situation is further exacerbated by diabetogenic immunosuppression, as tacrolimus causes direct islet toxicity and sirolimus can lead to insulin resistance (Lopez-Talavera, Garcia-Ocana et al. 2004). Therefore, the engraftment of a marginal mass of islets with withdrawal of exogenous insulin may

precipitate beta cell exhaustion and lead to chronic glucose toxicity, which causes further exacerbation of beta cell exhaustion. Thus, it may be that trying to achieve insulin independence is one of the main reasons for the high rate of attrition of islet grafts. Therefore, reduction of exogenous insulin after transplantation should probably be limited, in order to abolish hypoglycaemia, and then the transplanted islets should be left to smooth glycaemic control where the exogenous insulin release does not match the variable pattern of glucose metabolism (figure 8.1).

Figure 8.1: Schematic insulin profiles in normal individuals, exogenous insulin administration and after islet transplantation. Panel A shows the insulin profile of a normal individual with 3 biphasic peaks of insulin secretion, related to regular meals. Panel B shows the insulin profile of a type 1 diabetic maintained on a standard 4 times a day insulin delivery. The profile is superimposed on a normal insulin profile. Panel C is the insulin secretion (red area) required from an islet graft in a patient independent of insulin after islet transplantation. Panel D shows the reduced insulin secretion (red area) required from an islet graft post transplantation, if the patient is maintained on small doses of exogenous insulin.



This regime would reduce the glucose toxicity and high rate of insulin production enforced on transplanted islets after the withdrawal of insulin and hopefully lead to a longer islet graft survival while maintaining immaculate glycaemic control. After all, it is immaculate glycaemic control which delays the onset of complications (DCCT 1993) and may reverse secondary complications (Navarro, Sutherland et al. 1997; Fioretto, Steffes et al. 1998). Therefore, the aim of islet transplantation should probably be long term and lasting tight glycaemic control, irrelevant of administered exogenous insulin, rather than measuring success by insulin independence.

8.4.6 Conclusions

We have shown that human islet isolation and transplantation can be established de-novo and that good short term clinical transplant results can be achieved. The process of islet isolation and transplantation is not fully optimised but requires further modification to achieve better insulin independence rates with a lower incidence of complications.

8.5 Overall conclusions

Islet transplantation offers a promising treatment for patients with the severe complications of type 1 diabetes but is currently limited by the number of pancreases required for successful transplantation and the poor long term insulin independence rates. Islet engraftment probably holds the key to solving the problems associated with islet transplantation and hopefully, the ability to achieve success rates comparable to whole organ transplantation. Revascularisation is the first step in islet engraftment and I have developed a robust model for observing and quantifying this complex process which can be applied to strategies for improving islet engraftment. Desferrioxamine is a simple technique for preconditioning islets to over express the important angiogenic growth factor VEGF for prolonged periods without detrimentally affecting the viability or function of islets. Although I was unable to show positive benefit when DFO treatment was transferred to an in-vivo model, it remains a promising technique for hypoxia preconditioning which could be applied to islets and other organs for transplantation. Clinical islet transplantation is achievable within the United Kingdom but requires extensive training and has massive implications for resources and funding. The transfer of the Edmonton protocol to other units is achievable but once established, further optimisation of isolation and transplant protocols are required to improve outcome. The greatest hurdle facing islet transplantation in the UK is likely to be funding and the rationalisation of resources.

Appendix 1: Commonly used solutions

Unless stated, all chemicals were sourced from Sigma Aldrich, UK.

Collagenase

15 mg/ml collagenase P (Roche Diagnostics, Germany) in HBSS supplemented with 5.5mM calcium chloride solution
Made immediately prior to use and stored on ice

CMRL

CMRL culture medium (Gibco BRL) supplemented with
100 units/ml penicillin
0.1mg/ml streptomycin g
50mM HEPES (pH 7.4)
1.4mM L glutamine
10% foetal calf serum

Citrate buffer pH 6.0

7mM tri sodium citrate
25mM NaOH

GTE

1% glucose
25mM Tris/HCl pH7.5
10mM EDTA pH8.0

HBSS

Made from premixed powder in 1litre aliquots
Stored at 4°C for up to 2 weeks

HBSS /BSA

HBSS as above with 5mg/ml Bovine serum albumin (Roche Diagnostics, Germany)

Krebs Ringer solution

115mM	NaCl
4.7mM	KCl
5mM	NaHCO ₃
2.5mM	CaCl ₂
1.15mM	MgSO ₄
1.18mM	KH ₂ PO ₄
20mM	Hepes (pH 7.4)
2mg/ml	BSA

Make up to a final volume of 130ml with deionised water

LB Media

1% bacto-tryptone
0.5% bacto-yeast extract
171mM NaCl
Adjust to pH 7.0 with 5N NaOH
Sterilise by autoclaving

Lowry solution A

188mM Na₂CO₃
100mM NaOH
7mM Sodium Tartrate
1% SDS

Lowry solution B

160mM CuSO₄.5H₂O

Lysis buffer (for bacterial lysis)

0.2M NaOH

1% SDS

Lysis buffer #11 (for protein extraction)

50mM Tris (pH 7.4)

300mM NaCl

10% glycerol (w/v)

3mM EDTA

1mM MgCl₂

20mM β-glycerophosphate

25mM NaF

1% Triton X-100

Supplemented with Complete Protease Inhibitor cocktail (Roche Applied Science, UK)

M199

500ml of M199 culture media (Gibco BRL) supplemented with

100 units/ml penicillin

0.1mg/ml streptomycin g

50mM HEPES (pH 7.4)

1.4mM L-glutamine

10% (v/v) foetal calf serum

Neuraminidase V

Stock solution of 1U/ml neuramidase in

0.1M sodium acetate (pH 5.0)

10mM CaCl₂.

10 x TBE

0.34M Tris
0.45M Boric acid
10mM EDTA (pH 8.0)

TBS

137mM NaCl
3mM KCl
20mM Tris
pH to 7.4

TE (pH 7.4)

10 mM Tris HCl (pH 7.4)
1mM EDTA (pH 8.0)

Tris-EDTA (10x stock solution for antigen retrieval)

150mM Tris
10mM EDTA
20mM HCl
pH to 9 with NaOH

Appendix 2:

NICE guidance on pancreatic islet transplantation

NHS

Pancreatic islet cell transplantation

Issue date: **October 2003**

1 Guidance

1.1 Current evidence on the safety and efficacy of pancreatic islet cell transplantation does not appear adequate to support the use of this procedure without special arrangements for consent and for audit or research. Clinicians wishing to undertake pancreatic islet cell transplantation should inform the clinical governance leads in their trusts. They should ensure that patients offered it understand the uncertainty about the procedure's safety and efficacy and should provide them with clear written information. Use of the Institute's *Information for the Public* is recommended. Clinicians should ensure that appropriate arrangements are in place for audit or research. Publication of safety and efficacy outcomes will be useful in reducing the current uncertainty. NICE is not undertaking further investigation at present.

1.2 All cases should be registered with the International Islet Transplant Registry, which is based in Germany and run by Mathias D Brendel, Third Medical Department, University Hospital Giessen, D-35385 Giessen, Germany (www.med.uni-giessen.de/itr/).

2 The procedure

2.1 Indications

2.1.1 Pancreatic islet cell transplantation is used to treat type 1 diabetes mellitus and diabetes mellitus secondary to surgical removal of the pancreas for chronic pancreatitis. Type 1 diabetes mellitus is a condition, usually developing in childhood or early adulthood, in which the islet cells of the pancreas do not produce enough insulin, requiring the person to inject insulin. Chronic pancreatitis is a less common condition which often causes severe chronic abdominal pain. Sometimes pancreatitis is treated by removal of the pancreas (pancreatectomy). After a pancreatectomy, people are unable to produce insulin and injections are required.

2.2 Outline of the procedure

2.2.1 An alternative to insulin injection is pancreatic islet cell transplantation. This involves replacing a person's islet cells with either autologous islet cells (the person's own cells) or allogeneic islet cells (from a donor). The islet cells are usually placed in the liver.

2.2.2 Normal islet cells are able to produce naturally the correct amount of insulin. Pancreatic islet cell transplantation may, therefore, provide better continuous control of blood sugar than is achieved by insulin injections, which may in turn reduce diabetic complications. However, immunosuppressive drug treatment is needed to prevent rejection of transplanted allogeneic islet cells.

2.2.3 Other possible treatments for people suitable for pancreatic islet cell transplant include continuous insulin infusion using an automatic pump implanted under the skin, and total pancreatic transplantation.

2.3 Efficacy

2.3.1 Most of the evidence available was related to pancreatectomy. The rates of independence from injected insulin following the procedure were not always well reported. One study reported that 20 of 39 patients were insulin-independent at 1 month (51%).

2.3.2 The Specialist Advisors stated that if people were able to establish normal glucose control without insulin after transplantation then the potential benefits were likely to be great. However, the identified studies did not compare blood sugar control or risks of diabetic complications for

the treatment options (injected insulin versus pancreatic islet cell transplantation). There was also a lack of long-term follow-up data.

2.4 Safety

2.4.1 In one UK case series of 24 patients, complications were reported in three patients; these included partial portal vein and splenic thrombosis.

2.4.2 All of the Specialist Advisors reported that there was a potential risk of thrombosis of the portal vein, as well as of bleeding from the liver at the time of transplantation. They were also concerned about the side effects from the immunosuppressive drugs required after allogeneic transplantation, including malignancy.

2.5 Other comments

2.5.1 Most of the evidence related to pancreatectomy rather than to people with type 1 diabetes.

Andrew Dillon
Chief Executive
October 2003

3 Further information

3.1 NICE will review the guidance on pancreatic islet cell transplantation in December 2004, when research evidence is expected to be available.

Information for the Public

NICE has produced information describing its guidance on this procedure for patients, carers and those with a wider interest in healthcare. It explains the nature of the procedure and the decision made, and has been written with patient consent in mind. This information is available from www.nice.org.uk/IPG013publicinfoenglish and bilingually in English and Welsh from www.nice.org.uk/IPG013publicinfowelsh.

Sources of evidence

The evidence considered by the Interventional Procedures Advisory Committee is described in the following document.

Interventional Procedures Overview of Pancreatic Islet Cell Transplantation, March 2003.
Available from: www.nice.org.uk/IP071overview

Appendix 3: Local ethic committee submission

**ROYAL FREE HOSPITAL AND
MEDICAL SCHOOL ETHICS
COMMITTEE**

APPLICATION FORM Word 97

EC REF.....(leave blank)

SHORT TITLE OF PROJECT
(or your reference number)

Pancreatic Islet Transplantation

FULL TITLE

Clinical pancreatic islet transplantation in patients with insulin dependent diabetes.

CONTACT NAME & ADDRESS
(to be used in all correspondence relating to this application)

**Dr Martin Press, FRCP,
Consultant Physician,
Department of Endocrinology,
Royal Free Hampstead NHS Trust.**

SECTION 1

Details of Applicants

1. Responsible Consultant(s)

Name **Dr Martin Press** **Prof Stephen Powis**

Department	Endocrinology	Nephrology
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Name	Mr Keith Rolles	Miss Rozanne Lord
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Department	Surgery	Nephrology
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2. Name(s), qualification(s) and status of research worker(s) directly involved

Name Stephen Hughes Status Senior Research Scientist

Qualifications BSc, PhD Signature.....

Name	Mark Lowdell	Status	Head of Cellular Therapeutics
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Qualifications PhD Signature.....

3. Are other departments involved? *YES*

If yes, which: Radiology

Signatures of Head(s) of collaborating department(s) denoting approval

..... Department.....

..... Department.....

4. Who is sponsoring the study:

See Trust/Medical School research project registration sheet.

5. Aims and objectives of project

To ameliorate or correct Type 1 diabetes by the intraportal transplantation of isolated donor pancreatic islets.

6. Scientific background of study (*Approx 250 words*)

Metabolic control of insulin-dependent (Type 1) diabetes, sufficiently good to prevent complications such as blindness, amputations and renal failure, is not possible with conventional management without an unacceptably high risk of hypoglycaemia. Transplantation of the whole pancreas results in near-normal metabolic control and has been shown to reverse diabetic retinopathy and nephropathy, but it necessitates major surgery and carries with it a high morbidity due to the pancreatic digestive enzymes.

Transplantation of isolated pancreatic islets obviates these disadvantages, requiring only the intraportal injection of the isolated islets. A major breakthrough was reported three years ago from the University of Edmonton by Dr James Shapiro (N Engl J Med 2000; 343:230-8) who took advantage of a new immunosuppressive drug (rapamycin, sirolimus) to devise a steroid-free immunosuppressive regimen. Using this protocol, his group has now transplanted some 55 patients, and is reporting an 80% one year insulin independence rate (compared to 8% with 'conventional' immunosuppression).

At the Royal Free we have been developing the methodology of pancreatic digestion and islet purification and have now processed 32 pancreases. Our results are now good enough that we are in a position to do our first clinical transplant. Using this protocol, more than 200 successful transplants have now been performed world wide, and the technique is becoming an accepted clinical procedure.

SECTION 2 (continued)

Details of Project

7. Brief outline of project (*Approx 250 words*)

This application builds directly on a previously approved project (M218-2000) which has enabled us, as part of the UK Islet Transplant Consortium, to develop the methodology to separate pancreatic islets from donor pancreases obtained from brain dead cadaveric donors. Although this part of the programme was covered by an MREC protocol, considerable differences between the protocols of individual centres means that each centre is applying for its own LREC approval.

Donor pancreases will be retrieved under strict aseptic conditions under the supervision of Miss Rozanne Lord and Mr Keith Rolles and their respective organ procurement teams. The pancreases will be brought back to the Cellular Therapeutics Laboratory where they will be digested with collagenase and the islets purified under the strictest sterile conditions as per the previously approved protocol. The pancreatic islets will then be transplanted into the portal vein in the Radiology Department either transjugularly or percutaneously according to standard clinical methodology under local anaesthesia.

Recipients will receive an immunosuppressive regimen identical to that used by Dr Shapiro. Induction therapy is with daclizumab, followed by maintenance immunosuppression with sirolimus and low dose tacrolimus. Anti-infectious agents will be used as appropriate according to standard management of immunosuppressed patients.

Patients may require more than one transplant in order to achieve the engraftment of sufficient islets to allow them to discontinue their insulin injections. Most patients in Edmonton have required two.

8. Study design (*e.g. cohort, case control, epidemiological analysis*)

If we and other centres are successful in replicating Dr Shapiro's technique (and we have no reason to suspect that we will not be), this technique will cease to be clinical research and will take its place as a standard clinical procedure alongside the transplantation of other organs.

SECTION 2 (continued)

Details of Project

9. Size of the study.

Will the study involve:

(a) Human Subjects?

Yes

i) How many patients will be recruited?

This is limited by the availability of donor pancreases and by funding issues. The trust has generously agreed to provide funding designed to cover the first three patients, and Diabetes UK has agreed to cover the clinical costs of the first 10 patients (in the whole country). We will be aiming to perform approximately 12 transplants per year thereafter, subject to funding issues being resolved.

ii) How many controls will be needed?

None

iii) What is the primary end point?

Normalisation of metabolic control without the need for exogenous insulin injections

iv) How was the size of the study determined?

Practical considerations (see (i) above)

v) What is the statistical power of the study?

Not appropriate

(b) Patient Records?

No

i) How many records will be examined?

ii) How many control records will be examined?

iii) What is the primary end point?

iv) How was the size of the study determined?

v) What is the statistical power of the study?

10. Independent Review

Has the study been externally independently reviewed

No

If *yes* state which body individual has undertaken the review.....

If *no* you must obtain the signature of a person who has reviewed the study and who can guarantee its potential usefulness, either on increasing scientific knowledge or enhancing therapeutic power.

NB The person must be wholly independent of the study (i.e. not the head of department responsible) and must have the requisite experience and status to perform the task

Signature.....Name.....Date.....

Status.....

SECTION 3

Recruitment of Subjects

11. How will the subjects in the study be:

- i) **selected?** According to criteria agreed within the UK Islet Transplant Consortium. These give priority to patients with recurrent episodes of severe hypoglycaemia, particularly if hypoglycaemic warning symptoms are deficient, and to patients who have diabetic complications which are progressing despite the best metabolic control achievable with conventional insulin injections.
- ii) **recruited?** From diabetic out-patient clinics at the Royal Free.
- iii) **what inclusion criteria will be used?** **Patients aged 18-65 with C-peptide negative insulin dependent diabetes.**
- iv) **what exclusion criteria will be used?** Impaired renal or liver function; myocardial infarct within past 6 months; neoplasm within past 5 years; steroid use or other cause of insulin resistance; pregnancy or intended pregnancy; active hepatitis, HIV, tuberculosis or other chronic infection; evidence of likely non-compliance with medications or management.

How will the control subjects be: N/A

- i) **selected?**
- ii) **recruited?**
- iii) **what inclusion criteria will be used?**
- iv) **what exclusion criteria will be used?**

12. Will there be payment to research subjects of any sort?

No

SECTION 4

Consent

-
13. **Is *written* consent to be obtained?** **Yes**

If yes, please attach a copy of the consent form to be used

If no written consent is to be obtained, please justify

14. **How long will the subject have to decide whether to take part in the study?**

Likely to be many weeks in most cases.

15. **Will the subject be give a written information sheet or letter?** **Yes**

If yes please attach a copy to this application form

If no, please justify

16. **Have any special arrangements been made for subjects for whom English is not a first language?** **No**

If yes, give details:

If no, please justify: A high degree of understanding and co-operation is essential for the success of this project. Patients must be able to follow and comply with complicated instructions.

17. **Will any of the subjects be from one of the following vulnerable groups?**

Children under 18
People with learning difficulties
Unconscious or seriously ill
Psychiatric patients
Elderly patients
Other vulnerable groups

No

If yes, please justify

What special arrangements have been made to deal with the issue of consent for the subjects above?

SECTION 5

Details of intervention

-
- 18. Substances to be given to the subjects** (Special diets, drugs, isotopic tracers etc. State dose, and mode and frequency of administration)

Daclizumab, 1mg/kg iv every 14 days for 5 doses
Sirolimus, 0.2 mg/kg loading dose, then 0.1 mg/kg/day adjusted according to blood levels to give 12-15 ng/ml for the first 3 months and then 7-10 ng/ml.
Tacrolimus, 1 mg bd, titrated to give trough levels of 3-6 ng/ml.
Co-Trimazole, 480 mg/day for 3-6 months

-
- 19. Does the study involve the use of a new medicinal product or medical device or an existing product outside the terms of its product licence?**

Yes

If yes has a pharmaceutical or other commercial company arranged this trial and obtained a Clinical Trial Certificate or Clinical Trial Exemption?

No

Or have you obtained approval of the licensing authority by means of a DDX?

Yes

DDX or CTX Number.....(Please attach a copy)

-
- 20. Will any ionising or radioactive substances or X-Rays be administered?**

Yes

If yes please fill in Annex A

-
- 21. Please list those procedures in the study to which subjects will be exposed indicating those which will be part of normal care and those that will be additional (e.g. taking more samples than would otherwise be necessary). Please also indicate where treatment is withheld as a result of taking part in the project.**

Portal vein catheterisation, either percutaneous or transjugular is outside normal diabetic care. The procedure may need to be repeated more than once to obtain a sufficient mass of engrafted islets.

SECTION 6

Risks and ethical problems

- 22. Are there any potential hazards?** **Yes**

If yes, please give details, and give the likelihood of occurrence. Also give details of precautions taken to meet them, and arrangements to deal with adverse events.

Portal vein catheterisation is associated with a risk of haemorrhage or thrombosis, but the procedure is in routine use at this hospital and we would regard the risks as extremely small. Immunosuppression carries with it an increased risk of infections, and infection-related neoplasia, as with any other clinical transplant procedure. Sirolimus tends to cause mouth ulcers during the first weeks when the dose is high. Since it is still a relatively new drug, its long term risks remain unknown. Tacrolimus may cause nephrotoxicity.

- 23. Is this study likely to cause discomfort or distress?** **Yes**

If yes, please justify and give details

The portal vein catheterisation, which is performed under local anaesthetic, may be associated with slight discomfort.

- 24. Are there any particular ethical problems or considerations that you consider to be important or difficult with the proposed study?** **No**

If yes, please give details

- 25. Will information be given to the patient's General Practitioner?** **Yes**

Please note: permission should always be sought from research subjects before doing this

If yes, please enclose a copy of the information sheet for the GP

If no, please justify

- 26. If the study is on hospital patients, will consent of all Consultants whose patients are involved in this research be sought?** **Yes**

SECTION 7

Indemnity and Confidentiality

27.	<p>Is this NHS sponsored research to which HSG (96) 48 applies?</p> <p style="text-align: right;"><i>Yes</i></p> <p><i>or</i></p> <p>Is this research sponsored by the medical school for which indemnity is provided by an insurance policy taken out by the medical school?</p> <p style="text-align: right;"><i>No</i></p> <p><i>or</i></p> <p>Is this pharmaceutical company sponsored research, the company providing indemnity to the most recent ABPI guidelines?</p> <p style="text-align: right;"><i>No</i></p> <p><i>If yes please enclose a copy of the indemnity form and state</i></p> <p>The maximum indemnity per patient (ideally over</p>
	<p>£2million). Maximum Indemnity £.....</p> <p><i>or</i></p> <p>Is indemnity being provided in some other way</p> <p style="text-align: right;"><i>No</i></p> <p><i>If yes please specify</i></p>

28.	<p>In the case of equipment or medical devices will indemnity be provided by the manufacturer to the level specified in ABHI (Association of British Health-Care Industries) guidelines.</p> <p style="text-align: right;"><i>N/A</i></p> <p><i>If no please justify</i></p>
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29.	<p>Will the study data be retrieved from computer?</p> <p style="text-align: right;"><i>No</i></p> <p>Will the study data be held on computer?</p> <p style="text-align: right;"><i>Yes</i></p> <p>If, yes, will the Data Protection Act (1998) be followed?</p> <p style="text-align: right;"><i>Yes</i></p>
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30.	<p>Will the study include Audio/video recording?</p> <p style="text-align: right;"><i>No</i></p> <p><i>If yes (1) has specific consent been obtained?</i></p> <p style="text-align: right;"><i>Yes No</i></p> <p><i>(2) what will happen to the recordings at the end of the study?</i></p>
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31.	<p>Will medical records be examined by research workers outside the employment of the NHS?</p> <p style="text-align: right;"><i>No</i></p> <p><i>If yes, please ensure that this is made clear to the patient on the information sheet.</i></p>
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**PLEASE ENSURE THAT YOU COMPLETE THE CHECKLIST AND
ENCLOSE ALL RELEVANT DOCUMENTS**

DECLARATION

The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

I agree to supply interim reports on an annual basis and a final report, and to advise the ethics committee of any adverse or unexpected events that may occur during this project.

The research for which I am seeking ethical approval will be undertaken in no more than four LREC's geographical boundaries (applications involving five or more centres should be sent to a multi-centre research ethics committee - see health service guidelines HAG(97)23).

Signature of Responsible Consultant.....

Date.....

FOR ETHICS COMMITTEE USE

First Meeting.....

Applicant interviewed

Approved without changes

Approved after revision

Rejected

Signature of Chairman..... Date of Approval.....

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